



# BIOCHEMICAL STUDIES ON THE WHOLE AND FRACTIONATED THYMUS OF RATS INJECTED WITH $\beta$ -CHLOROETHYL VESICANTS\*

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(Received for publication, June 9, 1948)

The sulfur and nitrogen mustards have a selective destructive action on lymphoid tissue (1-3). Assessment of the cellular damage caused by these compounds is based chiefly on histologic evidence. The thymus shrinks in volume owing to the destruction of lymphocytes, inhibition of mitosis, and the migration of lymphocytes into the blood stream (3). The present investigation is concerned with (a) the changes produced in the cholesterol, phospholipide, and nucleic acid of the whole thymus and (b) changes in the total lipide, cholesterol, and phospholipide of the stroma, lymphocyte nuclei, and saline-soluble proteins of the thymus after injection of  $\beta$ -chloroethyl vesicants.

## Methods

Inbred male rats of Wistar stock, about 70 days old, were used as experimental animals. The agents and doses ( $LD_{100}$ ) used were as follows: (a) bis( $\beta$ -chloroethyl)sulfide (H), 0.4 mg. per kilo; (b) ethylbis( $\beta$ -chloroethyl)amine hydrochloride (HN1), 0.4 mg. per kilo; (c) methylbis( $\beta$ -chloroethyl)amine hydrochloride (HN2), 1.2 mg. per kilo; (d) tris( $\beta$ -chloroethyl)amine hydrochloride (HN3), 0.6 mg. per kilo. The nitrogen mustards were dissolved in saline and H was dissolved in thiodiglycol immediately before injection. The agents, dissolved in 0.2 to 0.3 ml. of solvent, were injected into the exposed jugular vein of the animal anesthetized with sodium pentobarbital. Control animals were injected with saline. All rats were fasted after injection. At varying intervals after injection the rats were exsanguinated under anesthesia and the thymus was removed immediately, weighed, and prepared for analysis.

Methods for analyzing the cholesterol, phospholipide, and nucleic acid in the intact thymus are outlined. The total cholesterol was determined after the thymus had been dried to constant weight *in vacuo* over  $P_2O_5$ . The dried tissue was cut into small pieces with sharp scissors, transferred

\* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

to an extraction thimble, and extracted under a reflux for 3 hours with 20 ml. of an absolute alcohol-acetone mixture (1:1). The extract was filtered and brought to 25 ml. with alcohol-acetone mixture. Aliquots were analyzed for total cholesterol by the Schoenheimer-Sperry procedure as modified by Sperry and Brand (4). Phospholipide phosphorus was determined in the individual fresh thymus of another group of rats. This tissue was homogenized with 20 ml. of an absolute alcohol-acetone (1:1) mixture and extracted under a reflux for 4 hours. Experiments were done to show that phospholipides were completely extracted by this procedure. The extract was brought to 25 ml. and aliquots were evaporated close to dryness on a hot-plate and the residue was immediately extracted with petroleum ether and the phosphorus was analyzed by the method of King (5) as modified by Allen (6). In a separate group of animals nucleic acid was determined spectrophotometrically by a modification of the method of Schneider (7). The whole thymus of each experimental rat was homogenized with the aid of 2 ml. of ice-cold distilled water and diluted to 10 ml. 2 ml. of the well mixed homogenate were transferred to a centrifuge tube, 4 ml. of 7.5 per cent trichloroacetic acid were added, and the mixture was heated at 90° for 15 minutes. After cooling, the tube was centrifuged and the supernatant was diluted 10 to 100 times with water, depending on the original thymus weight. Since the maximum absorption of nucleic acid dissolved in dilute trichloroacetic acid was at 260 m $\mu$ , all readings were made at this wavelength in a Beckman spectrophotometer. The nucleate content of a thymus extract was determined with the aid of a standard curve prepared with known amounts of calf thymus nucleate treated in the same manner as the unknowns.

Fractionation procedures were developed in this laboratory (8) to obtain stroma, lymphocyte nuclei, and saline-soluble proteins of the thymus. In order to obtain sufficient thymus (7 to 10 gm.) to yield adequate amounts of these three fractions in each group, it was necessary to sacrifice thirty to forty control rats, and 90 to 100 HN3-injected animals. The fractions were dried to constant weight *in vacuo* over P<sub>2</sub>O<sub>5</sub>. The dried materials, which were ground to a powder, were first extracted with absolute ethanol overnight under a reflux, followed by a 6 hour extraction with absolute ethanol-ether (1:1). These extracts were combined and brought to 100 ml. with the ethanol-ether mixture. Total cholesterol (4), total lipid carbon (9), and phospholipide phosphorus (5, 6) were determined on aliquots of this extract.

The following conversion factors were used: lipid P  $\times$  23.7 = phospholipide, phospholipide  $\times$  0.647 = phospholipide carbon, and cholesterol  $\times$  0.839 = cholesterol carbon. The combined cholesterol and phospholipide carbon values were subtracted from total lipid carbon to give the concentration of "neutral fat" carbon.

## Results

*Intact Whole Thymus*

*Cholesterol (Table I)*—The data for the mean values of dry weights of the individual thymuses and the percentage concentrations and contents of cholesterol after intravenous injection of saline and  $\beta$ -chloroethyl vesicants are summarized.

TABLE I

*Dry Weights and Cholesterol Concentrations and Contents of Thymus of Fasting Rats after Intravenous Injection of Saline and Mustards*

Days after injection	Saline	HN1	HN2	HN3	H
Mg. dry weight thymus per 100 gm. rat					
1	57 $\pm$ 3.7 (10)	39 $\pm$ 1.8* (10)	43 $\pm$ 1.4* (10)	45 $\pm$ 2.2* (10)	40 $\pm$ 1.6* (10)
2	46 $\pm$ 2.5 (10)	31 $\pm$ 1.9* (21)	43 $\pm$ 1.7* (9)	29 $\pm$ 2.1* (10)	32 $\pm$ 2.8* (10)
3	44 $\pm$ 2.4 (12)	31 $\pm$ 1.4* (12)	21 $\pm$ 1.7* (12)	24 $\pm$ 1.3* (10)	23 $\pm$ 2.1* (8)
4	40 $\pm$ 1.9 (18)	34 $\pm$ 2.6 (9)	23 $\pm$ 1.2* (10)	19 $\pm$ 1.9* (10)	23 $\pm$ 1.7* (7)
5	36 $\pm$ 2.2 (10)	25 $\pm$ 2.2* (11)		17 $\pm$ 2.0* (11)	27 $\pm$ 4.1 (8)
Mg. cholesterol per 100 gm. dry thymus					
1	1001 $\pm$ 37.7	1145 $\pm$ 45.6	1140 $\pm$ 44.6	1259 $\pm$ 50.0*	1297 $\pm$ 29.9*
2	1187 $\pm$ 66.8	1181 $\pm$ 66.7	1941 $\pm$ 104.1*	1627 $\pm$ 144.3*	1660 $\pm$ 120.5*
3	1184 $\pm$ 38.3	1988 $\pm$ 148.4*	2535 $\pm$ 150.8*	2039 $\pm$ 125.0*	2292 $\pm$ 118.5*
4	1546 $\pm$ 55.6	1484 $\pm$ 94.0	2641 $\pm$ 203.0*	2816 $\pm$ 246.0*	2411 $\pm$ 375.0
5	1622 $\pm$ 59.7	1891 $\pm$ 250.4		4151 $\pm$ 151.7*	2191 $\pm$ 310.2
Mg. cholesterol per 100 gm. rat					
1	0.56 $\pm$ 0.04	0.45 $\pm$ 0.03	0.49 $\pm$ 0.02	0.56 $\pm$ 0.03	0.52 $\pm$ 0.02
2	0.54 $\pm$ 0.01	0.54 $\pm$ 0.02	0.61 $\pm$ 0.03	0.44 $\pm$ 0.20	0.50 $\pm$ 0.03
3	0.52 $\pm$ 0.03	0.61 $\pm$ 0.03	0.53 $\pm$ 0.04	0.48 $\pm$ 0.03	0.52 $\pm$ 0.03
4	0.60 $\pm$ 0.02	0.49 $\pm$ 0.02*	0.60 $\pm$ 0.02	0.50 $\pm$ 0.02	0.47 $\pm$ 0.04*
5	0.58 $\pm$ 0.02	0.47 $\pm$ 0.03*		0.65 $\pm$ 0.05	0.53 $\pm$ 0.05

The figures in parentheses represent the number of animals in each group.

\* Represents significant difference from saline control.

Fasting causes an appreciable decrease in the thymus weight. The nitrogen and sulfur mustards are responsible for significant decreases in weight when compared with the saline controls. The greatest decreases are seen in the HN3 group.

The percentage concentrations of the cholesterol in dried thymus increase markedly in both the control saline and mustard groups. The remarkably



constant values for the cholesterol content per 100 gm. of rat indicate a close relationship between the loss in thymus weight and the increased percentage concentration of cholesterol.

*Phospholipide (Table II)*—The data for the phospholipide concentration and content of the thymus (fresh) after injection of saline and HN3 are shown. The number of animals in each group is small and the standard errors, which are omitted, are relatively large. Although the percentage concentrations of phospholipide are consistently greater in the HN3 groups, there is no significant statistical difference from the saline controls. The decrease in the phospholipide content is statistically significant and is associated with the resulting marked involution after HN3.

*Nucleic Acid (Table III)*—Fasting of the control animals does not affect the percentage concentration of nucleic acid but does cause an appreciable decrease in the content of nucleic acid. The most marked decreases in

TABLE II

*Percentage Concentrations and Contents of Phospholipide of Thymus of Fasting Rats after Intravenous Injection of Saline and HN3*

Days after injection	Mg. per 100 mg. rat thymus		Mg. per 100 gm. rat	
	Saline	HN3	Saline	HN3
2	1.39 (5)	1.62 (5)	2.8	1.6
3	1.35 (5)	1.65 (5)	2.5	1.1
4	1.49 (8)	1.84 (7)	1.8	0.9
5	1.26 (5)	1.34 (7)	1.6	1.0

The figures in parentheses represent the number of animals in each group.

both concentration and content of nucleic acid are observed after HN2 and HN3. Although HN1 is the most toxic of these agents, it causes the smallest change.

### *Thymus Fractions*

*Percentage Distribution (Fig. 1)*—The percentage distribution of stroma, lymphocyte nuclei, and the saline-soluble proteins or cytoplasmic constituents of dry thymus at various periods after fasting and after the injection of saline or HN3 is shown. From these data it is clear that the increase in the stroma fraction is roughly proportional to the decrease in lymphocytes; the cytoplasmic components are not appreciably changed by the experimental procedures. A 4 day period of fasting in the saline group is responsible for a decrease in lymphocytes from 65 to 20 per cent; the value on the 4th day for the HN3 group is 3 per cent.

TABLE III

*Nucleic Acid Concentrations and Contents of Thymus of Fasting Rats after Intravenous Injection of Saline and Mustards*

Days after injection	Saline	HN1	HN2	HN3
Mg. nucleic acid per 100 mg. thymus				
1	2.14 $\pm$ 0.06 (9)	2.18 $\pm$ 0.06 (15)	1.45 $\pm$ 0.11* (10)	2.04 $\pm$ 0.04 (10)
2	2.20 $\pm$ 0.03 (10)	1.87 $\pm$ 0.08* (16)	1.31 $\pm$ 0.08* (11)	1.59 $\pm$ 0.08* (10)
3	2.13 $\pm$ 0.12 (10)	1.77 $\pm$ 0.07* (13)	1.05 $\pm$ 0.07* (9)	1.48 $\pm$ 0.04* (10)
4	1.97 $\pm$ 0.21 (10)	1.96 $\pm$ 0.07 (10)	1.28 $\pm$ 0.04* (10)	1.09 $\pm$ 0.05* (10)
5	1.85 $\pm$ 0.17 (10)	1.66 $\pm$ 0.10 (10)	1.16 $\pm$ 0.12* (10)	1.19 $\pm$ 0.08* (14)
Mg. nucleic acid per 100 gm. rat				
1	4.10 $\pm$ 0.18	3.48 $\pm$ 0.20	2.10 $\pm$ 0.15*	2.69 $\pm$ 0.14*
2	4.07 $\pm$ 0.14	2.27 $\pm$ 0.16*	1.47 $\pm$ 0.09*	1.44 $\pm$ 0.15*
3	3.60 $\pm$ 0.25	1.88 $\pm$ 0.13*	0.84 $\pm$ 0.08*	0.94 $\pm$ 0.07*
4	2.33 $\pm$ 0.21	2.16 $\pm$ 0.27	0.99 $\pm$ 0.06*	0.56 $\pm$ 0.04*
5	2.05 $\pm$ 0.17	1.49 $\pm$ 0.34*	0.93 $\pm$ 0.11*	0.76 $\pm$ 0.08*

The figures in parentheses represent the number of animals in each group.

\* Represents significant difference from saline control.

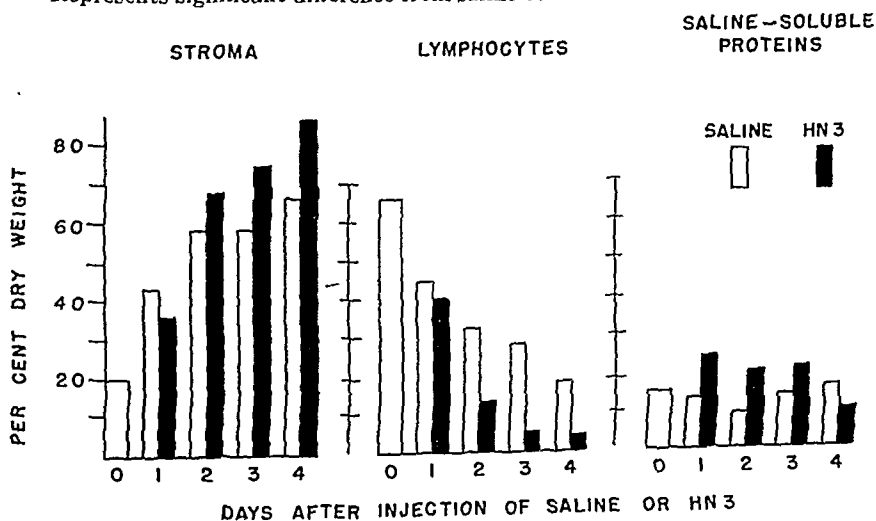


FIG. 1. Percentage distribution of stroma, lymphocytes, and saline-soluble proteins in the thymus of fasting rats injected with saline or HN3.

### *Lipide Analyses*

*Stroma* (Fig. 2)—The concentration of total lipid carbon varies considerably in both groups owing to the neutral fat component. These vari-

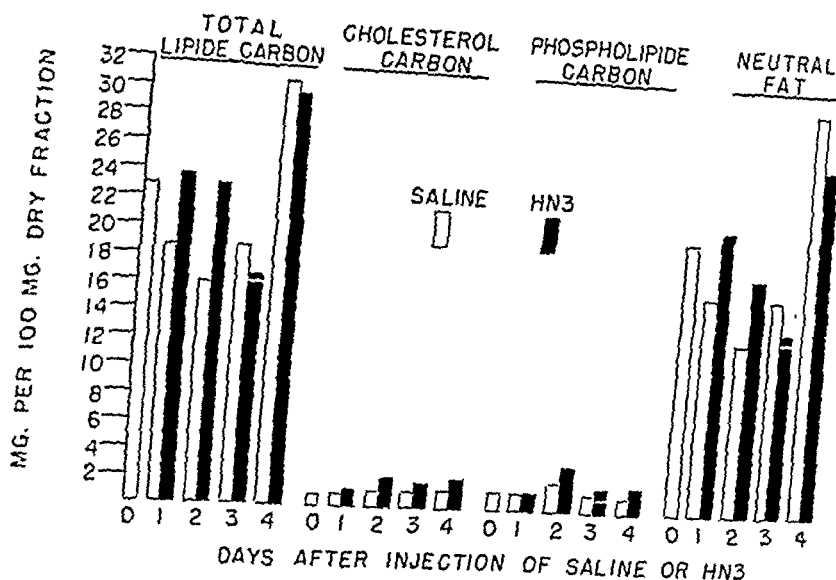


FIG. 2. Percentage concentration of total lipid, cholesterol, phospholipide, and neutral fat carbon in stroma of the thymus of fasting rats. Breaks in the graph represent two analyses on different samples.

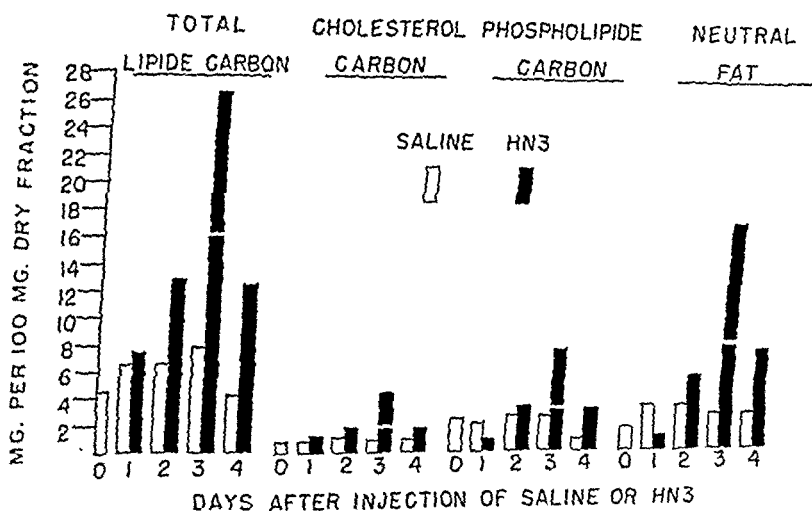


FIG. 3. Percentage concentration of total lipid, cholesterol, phospholipide and neutral fat carbon in lymphocyte nuclei of the thymus of fasting rats.

ations do not appear to be attributable to the effect of HN3. Both the cholesterol and phospholipide concentrations of the HN3 groups increase appreciably above most of the saline controls.

*Lymphocyte Nuclei (Fig. 3)*—Fasting in the saline-injected groups caused minor variations in the concentrations of the lipid components. After in-

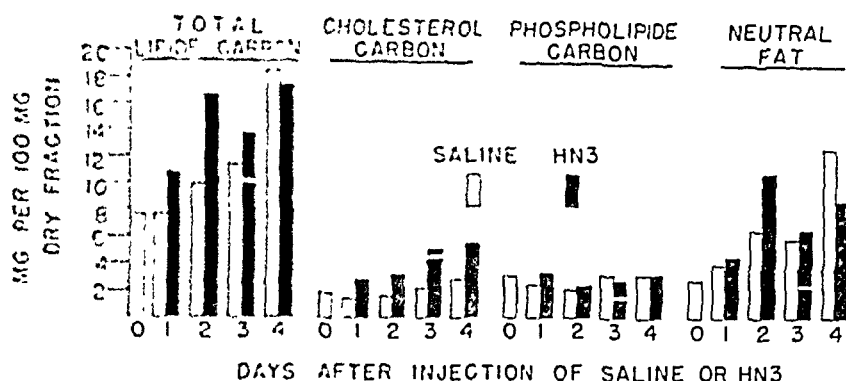


FIG. 4. Percentage concentration of total lipid, cholesterol, phospholipide, and neutral fat carbon in saline-soluble proteins of the thymus of fasting rats.

TABLE IV

*Distribution of Cholesterol in Fractions of 100 Gm. of Dried Thymus of Fasting Rats after Saline and HN3 Injection*

Days after injection		Saline		HN3	
		mg.	per cent	mg.	per cent
0	Stroma	193	18		
	Lymphocyte nuclei	535	50		
	Saline-soluble constituents	312	32		
		1070			
1	Stroma	410	41	396	23
	Lymphocyte nuclei	390	39	516	30
	Saline-soluble constituents	200	20	809	47
		1000		1721	
2	Stroma	741	60	1650	62
	Lymphocyte nuclei	310	25	265	10
	Saline-soluble constituents	186	15	742	28
		1240		2657	
3	Stroma	857	55	1600	56
	Lymphocyte nuclei	296	19	114	4
	Saline-soluble constituents	405	26	1140	40
		1558		2854	
4	Stroma	1090	59	2140	73
	Lymphocyte nuclei	185	10	88	3
	Saline-soluble constituents	574	31	701	24
		1849		2929	

jecting HN3, the total lipide concentration increases markedly on the 2nd day and reaches a maximum on the 3rd day. These increases are chiefly due to the increases in neutral fat. The values for cholesterol and phospholipide are consistently greater than those for the saline groups.

*Saline-Soluble Components (Fig. 4)*—This fraction contains the saline-soluble proteins and suspended particulate material of the cytoplasm. Fasting for 4 days is responsible for an impressive increase in lipide carbon from 7.7 to 18.6 per cent, owing chiefly to increases in neutral fat carbon; in the HN3 groups appreciable increases are noted on the 1st and 2nd days. The cholesterol concentrations in the nitrogen mustard groups are consistently increased about 100 per cent above the values of the saline controls. The phospholipide concentrations are not appreciably affected.

*Cholesterol Distribution (Table IV)*—The concentration and distribution of cholesterol in the three thymus fractions of rats injected with saline and HN3 are shown. This analysis yields information showing the fractions responsible for the constancy of the cholesterol values seen in these involuted glands (Table I). In both the saline and HN3 groups the cholesterol concentration of the stroma fraction increases from 18 to a maximum of 73 per cent of the total thymus. Concurrently the cholesterol content of the lymphocyte nuclei decreases from 50 to as low as 3 per cent of the total. The changes in the cholesterol content of the cytoplasmic fraction, particularly in the HN3 groups, are not markedly changed. The sums of the cholesterol content of the three fractions per 100 gm. of thymus increase in both the saline and HN3 groups and approximate closely the results shown for the whole thymus in Table I.

#### DISCUSSION

Despite the pronounced involution of the thymus, due chiefly to the loss of lymphocytes, the percentage concentration of cholesterol increases and the cholesterol content of the gland remains constant. The cholesterol of the lymphocyte nuclei of the control thymus accounts for 50 per cent of the total cholesterol; after HN3 injection, the lymphocytes account for 3 per cent of the total cholesterol of the involuted thymus. It is obvious that the stroma and the cytoplasm must contain the additional cholesterol to compensate for the deficit incurred by lymphocyte loss. It is therefore postulated that the thymus is capable of manufacturing a cholesterol-rich lipoprotein. It has been shown that the electrophoretic patterns and the lipide content of the plasma fraction II+III of the rat are changed after the injection of HN3.<sup>1</sup> It seems possible that some of the increased con-

<sup>1</sup> Chanutin, A., and Gjessing, E. C., unpublished results.

tent of lipoprotein of the plasma of injured animals originates in lymphoid tissue.

According to Kindred<sup>2</sup> the nuclei of the rat thymus lymphocytes show no morphologic changes during fasting. On the 1st day after injecting HN3, the largest number of degenerated lymphocytes is observed and a large proportion of the nuclei of surviving lymphocytes is partially lobulated. On subsequent days the number of degenerated cells decreases progressively, practically all of the lymphocyte nuclei are irregular in shape, but the distribution and appearance of the chromatin are normal. No correlation is apparent between the cytologic observations and the changes in the total lipid concentration of the nuclei. The very marked and sudden increases in the neutral fat concentration of the nuclei on the 2nd, 3rd, and 4th days after HN3 indicate extensive chemical alterations. This may represent the type of lipid infiltration commonly seen in cells after many types of chemical injury. A study of the literature has failed to disclose information dealing with the quantitative aspects of lipides of nuclei before and after injury.

#### SUMMARY

The effect of the intravenous injection of saline and tris( $\beta$ -chloroethyl)-amine (HN3) upon the total cholesterol, phospholipide, and nucleic acid concentration of the thymus of the fasting rat is presented. The percentage concentration of cholesterol increases but the cholesterol content of the involuted thymus remains constant. The phospholipide and nucleic acid contents decrease.

Data are presented for the changes in the total lipid, cholesterol, and phospholipide carbon concentrations of the stroma, lymphocyte nuclei, and cytoplasmic constituents of the thymus after injection with saline and HN3. The pronounced effect of nitrogen mustard on decreasing the number of lymphocytes is demonstrated. HN3 causes a marked increase in the neutral fat of the lymphocytes, which is suggestive of lipid infiltration. The cholesterol concentration of each of the three fractions is elevated after HN3. The phospholipide concentration is slightly elevated in the stroma and lymphocyte nuclei.

Lymphoid tissue appears capable of synthesizing a cholesterol-rich lipoprotein.

The authors are indebted to Mr. Curtis S. Floyd, Miss Florence L. Jones, and Miss Elizabeth A. Lentz for technical assistance.

<sup>2</sup> Kindred, J. E., personal communication.

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# GLYCOLYSIS IN TUMOR HOMOGENATES\*

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(Received for publication, July 2, 1948)

Recent reports (1, 2) have strengthened the concept that tumor metabolism involves a very active glycolysis of the phosphorylating type. Suitable media for the study of glycolysis in homogenates of brain have been devised by Racker and Krimsky (3, 4) and Utter *et al.* (5, 6). These media were used, with slight modifications, by Novikoff *et al.* (2) for measurement of glycolysis in tumor homogenates. If the assumption that tumor uses high energy phosphate bonds as a source of energy for synthetic reactions is correct, then one important prerequisite for the study of synthetic reactions in tumor homogenates is the development of a medium which is optimal for sustaining phosphate bond energy in these homogenates. The object of this paper is to present data concerning the development of such optimal media for glycolysis in homogenates of Flexner-Jobling carcinoma. The medium for optimal glycolysis was developed not only on the basis of the  $Q_{CO_2}$ , but also in terms of lactate production, as determined chemically, and in terms of the esterification of inorganic phosphate.

## EXPERIMENTAL

Flexner-Jobling carcinoma transplants in Sprague-Dawley albino rats were used throughout.<sup>1</sup> These were taken for experiment at 8 to 12 days after subcutaneous transplantation, at which time they weighed 500 to 1000 mg. This material had the advantage that it was very reproducible and as free as possible from necrotic tissue or other tissue elements.

The tumors were rapidly excised from decapitated rats and placed in small beakers of isotonic KCl immersed in chopped ice. After all outer connective tissue had been trimmed off, the tumors were weighed and added to cold Potter-Elvehjem (7) homogenizer tubes and alkaline<sup>2</sup> isotonic KCl

\* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>1</sup> The author wishes to acknowledge the assistance of Mr. B. E. Kline, who provided the tumor transplants.

<sup>2</sup> After preliminary experiments which indicated very little difference between the results obtained with homogenates made in isotonic KCl and those made in water, with a slight superiority of the former, alkaline KCl was routinely used. This alkaline KCl was isotonic KCl to which had been added 8.0 ml. of 0.02 M  $KHCO_3$  per



net phosphorus uptake, in a 40 minute incubation, of varying tonicity in our optimal medium by additions of KCl.

*Initial pH of Medium*—In the medium, optimal in other respects, pH was varied by varying the bicarbonate concentration. The results are illustrated in Fig. 1. The values used are the initial pH before glycolysis has begun. The pH was checked after the gassing technique by rapidly removing the flasks and inserting a glass electrode in the contents. The pH values found agreed well with those predicted from calculations of the Henderson-Hasselbalch equation (9). The data in Fig. 1 are for a 40 minute incubation, during which separate experiments showed that the pH drop was 0.1 to 0.2 unit. The higher pH probably favors higher adenosinetriphosphatase activity. At lower than optimal magnesium concen-

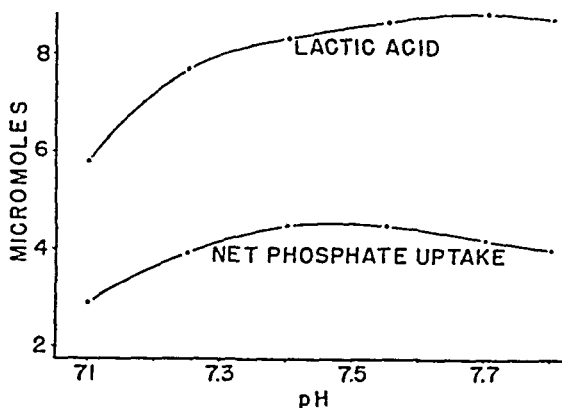


Fig. 1. Effect of initial pH of medium on lactic acid production and net phosphorus esterification per flask in a 40 minute incubation with 30 mg. of tissue.

trations, the range of optimal pH is much narrower, phosphorus uptake dropping sooner as pH progresses above 7.4.

*Potassium versus Sodium*—Boyer *et al.* (11) have discussed the necessity of potassium for phosphate transfers and demonstrated a potassium requirement for certain of these reactions. The substrates for our medium, the saline, bicarbonate, etc., were all potassium salts. Data provided in Table II demonstrate that potassium is necessary for optimal glycolysis, that it cannot be replaced completely by sodium without loss in efficiency, that the potassium level in the medium is sufficient, and that extra additions of potassium beyond 0.01 M do not alter the efficiency.

*Substrates*—Presumably the main substrate required for tumor glycolysis would be glucose. However, in operating a phosphorylating glycolysis, it is necessary first to phosphorylate the glucose. To permit this would

make necessary the addition of relatively large amounts of adenosine triphosphate (ATP), which would lead to inefficiency in a homogenate system because of the active ATPase. Hence it is more practical to start the reaction by addition of a small amount of hexose diphosphate<sup>3</sup> (HDP). When no ATP or HDP was added to the reaction mixture, no lactic acid (less than 0.2 micromole) or phosphoglyceric acid was produced. When small amounts of HDP were added, the HDP was quantitatively converted, at a very rapid rate, to the equivalent of phosphoglyceric acid. When both glucose and HDP were added, a large increase in the phosphoglyceric acid production occurred above that theoretically possible from the HDP,<sup>4</sup> and inorganic phosphate was esterified. Large amounts of HDP decrease the net phosphorus uptake, probably by permitting more phosphatase activity and possibly by permitting a reaction rate exceeding the rate of the glucose-phosphorylating enzymes. Data concerning the effects of varying the HDP concentration in the otherwise optimal medium are presented in

TABLE II  
*Potassium versus Sodium*

Experimental conditions	Lactic acid production per flask	Net P esterified per flask
	micromoles	micromoles
All sodium salts .....	2.6	-0.3
Potassium, 0.01 M; all other salts sodium ..	10.4	4.0
" salts entirely (K approximately 0.07 M) ..	10.5	4.1

Fig. 2. A 40 minute incubation period was used. Similarly, effects of varying glucose concentration with HDP optimal are shown in Table III. The amount of glucose glycolyzed is independent of concentration, under these conditions, to very low levels. This is probably not true *in vivo* (12) as the HDP level is very low (1).

*Pyruvate*—Pyruvate is added to act as hydrogen acceptor. This is necessary because the normal reactions which would provide it are blocked by the fluoride. However, when fluoride is absent, the system still derives

<sup>3</sup> Hexose diphosphate used in this study was prepared from HDP obtained as barium salt from the Schwarz Laboratories, Inc., New York, by precipitation as the acid barium salt. Before it was used, the barium was removed with sulfuric acid and the HDP neutralized with potassium bicarbonate.

<sup>4</sup> Production of lactic acid in the absence of added glucose exceeds the theoretical amount obtainable from added HDP to a small extent. A corresponding amount of pyruvic acid disappears from the lactic-pyruvic sum, indicating the possibility that a dismutation of 2 molecules of pyruvic to 1 of lactic, 1 of acetic, and 1 of carbon dioxide occurs. Direct evidence that this is the case has not yet been obtained.

some benefit from addition of a catalytic amount of pyruvate to prime the reaction. Measurements concerning the effects of adding or omitting pyruvate are presented in Table IV. The data for no pyruvate addition with fluoride present permit calculation of the extent to which the fluoride is inhibiting at the phosphoglyceric acid stage. The inhibition with 0.01  $M$  KF is indicated to be approximately 90 per cent.

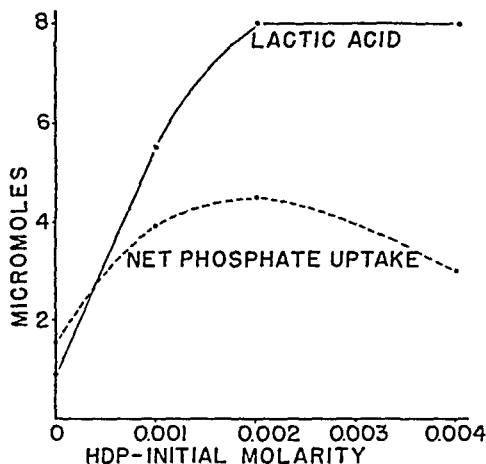


FIG. 2. Effects of varied HDP additions on micromoles of lactic acid produced and net phosphorus esterified per flask in a 40 minute incubation with 30 mg. of tissue.

TABLE III  
*Effect of Varied Glucose Concentration on Glycolysis*

Glucose concentration	Lactic acid produced per flask	Net P uptake per flask
$M$	<i>micromoles</i>	<i>micromoles</i>
0	7.6	0
0.0013	10.2*	3.9
0.01	10.4	4.2
0.02	10.1	4.2

\* This actually represents glycolysis of two-thirds of the added glucose.

*Magnesium and Fluoride*—In studies of these two ions, variations were made in both, since the concentrations being studied are such that the medium is close to or above saturation with magnesium fluoride. However, no indications were obtained that any precipitation was occurring. Table V illustrates the effect of variations in magnesium with fluoride optimal (0.01  $M$ ) and Table VI illustrates the effects of varied fluoride concentrations with magnesium constant (0.0066  $M$ ). The decision was made to use 0.01

M fluoride, despite higher phosphorylation efficiency with higher levels, because of the inhibiting effect on the glycolytic rate of such increases in fluoride.

TABLE IV  
*Effects of Added Pyruvate on Glycolysis in 40 Minute Incubation*

Conditions	Lactic acid produced per flask	Net P uptake per flask
	micromoles	micromoles
Complete medium .....	9.2	4.0
Minus pyruvate .....	0.9	-0.5
" " minus fluoride .....	8.0	-4.4*
" fluoride + 0.1 optimal pyruvate .....	10.0	-3.2
" " + optimal pyruvate .....	11.0	-3.2

\* The theoretically possible net P loss to the medium from the added HDP alone is 12 micromoles.

TABLE V  
*Effects of Varied Magnesium Concentration in 40 Minute Incubation*

Magnesium concentration	Lactic acid produced per flask	Net P uptake per flask
M	micromoles	micromoles
0	7.60	-0.23
0.00165	8.85	0.55
0.0033	8.85	1.95
0.0066	8.75	3.5
0.0099	8.2	3.5
0.0133	7.9	3.5

TABLE VI  
*Effects of Varied Fluoride Concentrations on Glycolytic Rate<sup>r</sup> During 40 Minute Incubation*

Potassium fluoride concentration	Lactic acid produced per flask	Net P uptake per flask
M	micromoles	micromoles
0	10.5	-10.5
0.0066	8.45	1.6
0.010	8.75	3.5
0.020	6.75	4.5
0.030	5.85	4.7

*Adenosine Triphosphate*—The adenosine phosphates are known to be coenzymes or "carriers" in the mechanism of enzymatic phosphate transfer. Experiments with adenylic acid and ATP, the former prepared by the

method of Kerr (13), the latter by the method of LePage (9), both in pure state, indicated that they were equivalent. A slightly higher phosphorus uptake is noted with adenylic acid. The effects of varying ATP concentration in the medium are illustrated in Fig. 3. Net phosphorus uptake tends to decrease at higher levels. This is undoubtedly due to increased ATPase activity at higher substrate levels.

*Diphosphopyridine Nucleotide (DPN)*—This compound is known to be required as coenzyme for hydrogen transfer in glycolysis. For use in our experiments it was prepared by the method of LePage (14) and further purified by solution in acid methanol and precipitation with ethyl acetate. This purification was employed to lower the content of adenylic acid (the

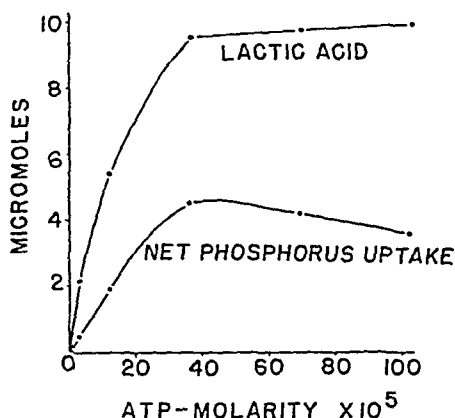


FIG. 3. Effects of varied ATP concentration on lactic acid production and net phosphorus esterification by 30 mg. of tissue in a 40 minute incubation.

chief impurity) in order to permit more quantitative definition of the ATP requirement. The preparations as used assayed 85 per cent DPN and contain less than 10 per cent of adenylic acid (without calculation for water of hydration). Fig. 4 illustrates the effects of varying additions of DPN.

*Protective Factors*—One compound cited as a protective factor for glycolytic enzymes is glutathione (15). Addition of glutathione to the medium had no effect upon duration or rate of glycolysis in our tumor homogenates.

Novikoff *et al.* (2) demonstrated that nicotinamide had a protective effect on the tumor homogenate system. Presumably this is due to its inhibitory effect on the nucleosidase breaking down DPN. Recently it has been shown that  $\alpha$ -tocopherol phosphate inhibits breakdown of DPN in various rat tissues (16) and that a pyrophosphatase rather than a nucleosidase is responsible for DPN breakdown in kidney (17). It was therefore of interest to determine the effects of both nicotinamide and  $\alpha$ -

(tocopherol phosphate<sup>5</sup> on preservation of DPN in this tumor homogenate system. Data on lactic acid production and phosphorus uptake, in a 40 minute incubation, are given in Table VII. DPN breakdown in these experiments was markedly inhibited by nicotinamide, but was unaffected by  $\alpha$ -tocopherol phosphate.

*Inorganic Phosphate*—Inorganic orthophosphate is obviously necessary in the phosphorylating glycolysis mechanism. A discussion of its possible rôle in regulation of rate was provided by Potter (18). It was of value in making accurate measurements of phosphate uptake to have the phosphate of the medium relatively low. It is necessary to reduce the phosphate of the medium to a very low level before it affects the glycolytic rate. Data

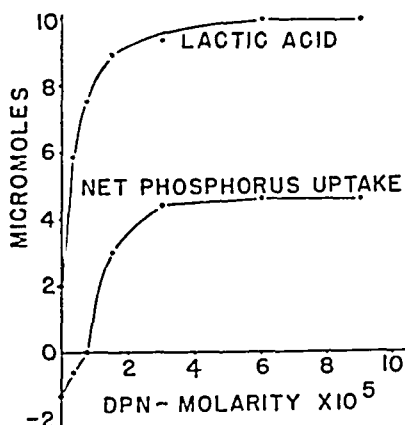


FIG. 4. Effects of varied DPN on lactic acid production and net phosphorus esterification by 30 mg. of tissue in a 40 minute incubation.

to illustrate this are provided in Table VIII. The lowest initial concentration listed there represents no phosphate addition, and is that resulting from the phosphorus present in the added tissue.

*Homogenate*—In all of the experiments described above, 30 mg., wet weight, of tumor were used per reaction vessel. This gives a suitable rate of reaction. Data provided in Fig. 5 for a 20 minute incubation illustrate that the lactic acid produced (phosphoglyceric) is proportional to the amount of tissue added. When incubation is carried beyond this time, the linear relationship fails because HDP becomes limiting. The phosphorylation of glucose with the phosphate bond energy gained from the triose phosphate dehydrogenase reaction is not 100 per cent efficient, owing to ATPase activity, and consequently the HDP concentration progressively

<sup>5</sup> The author wishes to thank Dr. Stanley Ames of Distillation Products, Inc., for a gift of  $\alpha$ -tocopherol phosphate.

TABLE VII

*Effects of Nicotinamide and  $\alpha$ -Tocopherol Phosphate in 40 Minute Incubation*

Conditions	Lactic acid production per flask	Net P uptake per flask
	<i>micromoles</i>	<i>micromoles</i>
Unprotected system.....	2.8	0.65
“ “ + $\alpha$ -tocopherol phosphate, 1 mg. per flask.....	3.15	0.68
Unprotected system + nicotinamide, 0.04 M.....	7.1	4.2

TABLE VIII

*Effect of Varied Inorganic Phosphate Levels in 40 Minute Incubation*

Initial phosphate concentration	Final phosphate concentration	Net P uptake per flask	Lactic acid produced per flask
$\mu \times 10^{-3}$	$\mu \times 10^{-3}$	<i>micromoles</i>	<i>micromoles</i>
0.25	0.12	0.37	3.2
0.37	0.11	0.82	3.4
0.53	0.14	1.1	3.6
0.80	0.14	1.9	4.6
1.34	0.32	3.0	8.1
2.7	1.2	4.2	9.0

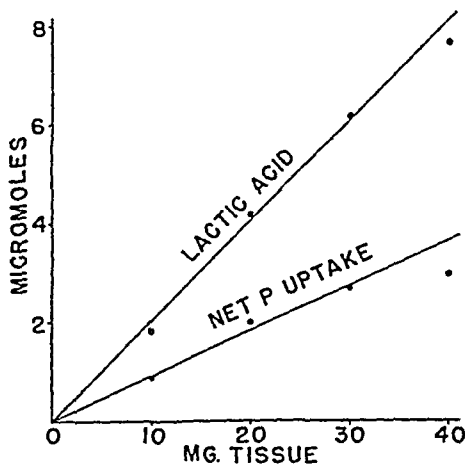


FIG. 5. Effects of varied additions of tissue on lactic acid production and net phosphorus esterification in a 20 minute incubation.

falls. During the first 20 minutes incubation, HDP has not become limiting, although more has been glycolyzed than was initially present (6.0 micromoles added).

The optimal medium finally selected had the following molar composition: potassium phosphate, 0.0024; potassium bicarbonate, 0.025; nicotinamide, 0.040; adenosine triphosphate,<sup>6</sup> 0.00033; diphosphopyridine nucleotide,<sup>6</sup> 0.00020; hexose diphosphate,<sup>6</sup> 0.0020; pyruvic acid,<sup>6</sup> 0.0050; magnesium chloride, 0.0066; potassium fluoride, 0.010; glucose, 0.010.

*Rate of Reaction*—The data in Fig. 6 illustrate the rate of reaction in typical experiments with optimal homogenate medium and 30 and 10 mg. of tissue. For some purposes it would be desirable to use only 10 mg. Nearly the same linearity of rate can be obtained with the higher tissue concentrations, however, if more HDP is added.

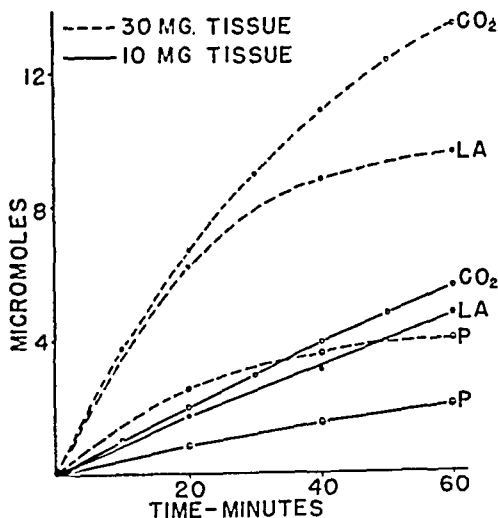


FIG. 6. Reaction rates in the optimal homogenate medium obtained with two tissue levels. CO<sub>2</sub> = carbon dioxide evolved from the bicarbonate buffer per flask; LA = lactic acid produced per flask; P = net phosphorus esterification per flask.

#### DISCUSSION

It was important to prove that high energy phosphorus in the form of ATP was present throughout the reaction period. Preliminary studies concerning this indicate that at least two-thirds of the ATP originally added is present as such after a 40 minute incubation. Further studies regarding the rates of transformation of the various phosphorylated intermediates will be made the subject of a later publication.

Some studies have been made concerning the glycolytic rates obtainable with homogenates of normal tissues. Of those examined, all appear to be able to glycolyze HDP very rapidly. Rat brain homogenate, in a medium similar to the one herein described, is able to glycolyze glucose and give

<sup>6</sup> Added as potassium salts.



large phosphorus uptakes. However, rat liver and kidney produce phosphoglyceric acid theoretically equivalent to the HDP added and do not glycolyze glucose or take up phosphorus in this medium. The study of conditions necessary to obtain phosphorylation of glucose in rat liver and kidney homogenates will be the subject of a later publication.

#### SUMMARY

With Flexner-Jobling rat carcinoma transplants as the tissue source, a medium has been devised for glycolysis in tumor homogenates. This medium permits glycolysis of glucose, esterification of inorganic phosphate, and maintenance of phosphate bond energy.

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# CENTRIFUGAL FRACTIONATION OF GLYCOLYTIC ENZYMES IN TISSUE HOMOGENATES\*

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(Received for publication, July 19, 1948)

Experiments on aerobic and anaerobic glycolysis in mammalian tissues have usually been conducted either with slices or with extracts. The latter have usually been made by breaking up the tissue and centrifuging and discarding some fraction of the particulate matter. Studies with slices have disadvantages in certain cases because of permeability factors. Results with extracts are frequently misleading because of variability in the amount of material discarded and lack of knowledge concerning the relation between the activity of the extract and that of the discarded portion. When reactions are studied for which information is not available as to intracellular distribution of the enzymes involved, it is advantageous to use whole homogenates. In this case permeability factors are largely ruled out and no part of the activity is discarded.

The complexities with regard to intracellular distribution of enzymes involved in glycolysis studies have been discussed by Meyerhof *et al.* (1-3). Recently a medium was devised for studies of anaerobic glycolysis with homogenates of Flexner-Jobling rat carcinoma, which enables these homogenates to maintain phosphate bond energy for reasonable periods of time and consequently permits studies of synthetic reactions with these homogenates (4). This medium was found to be approximately optimal for other tissues as well.

Centrifugal fractionations of tissue homogenates now permit separation of intracellular material into four well defined fractions (5): (a) a nuclear fraction, (b) the mitochondria or "large granule" fraction, (c) submicroscopic particle fraction, and (d) a supernatant fluid containing soluble enzymes. The object of this paper is to present the results of experiments in which homogenates were separated into these four fractions and recombined in the various possible combinations. Measurements were made of lactic acid production and net uptake of inorganic orthophosphate by the fractions.

\* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

## EXPERIMENTAL

Since the centrifugal fractionation procedure requires some 5 hours, and the glycolytic measurements cannot be taken until fractionation is completed, it was first necessary to study the stability of the enzyme systems. Data presented in Table I indicate that little decline in activity occurs in 5 hours at 0°, but that considerable decline occurs in 24 hours. It was therefore possible to complete the fractionation and make the glycolytic measurements before any significant fraction of the activity was lost.

For the study of enzyme distribution in tumor cells, Flexner-Jobling carcinoma transplants<sup>1</sup> were made in Sprague-Dawley rats and the tumors taken 8 to 12 days later, when they were actively growing and free from necrosis. Liver is a very suitable normal tissue for this fractionation procedure. It is composed mainly of one type of cell and provides clear cut

TABLE I

*Stability of Glycolytic System in Homogenates Stored at 0°*

Glycolysis obtained in 40 minutes with 30 mg. of tissue. The results are expressed in micromoles per flask.

Age of homogenate at 0° <i>hrs.</i>	Flexner-Jobling carcinoma		Rat liver		Rabbit liver	
	Lactic acid	Net P uptake	Lactic acid	Net P uptake	Lactic acid	Net P uptake
0	9.4	4.0	6.8	-2.8	6.4	0.6
5	9.0	3.6	6.2	-2.8	6.1	0.5
24	2.6	1.0	3.4	-3.0	3.0	-0.9

fractions. However, homogenates of liver tissue from intact, normal rats, though they glycolyze hexose diphosphate rapidly, do not appear to glycolyze glucose; they give inorganic phosphate output on our medium. Rabbit liver was found to be more suitable, and consequently it was used as an example of a normal, differentiated tissue. The rabbits used were New Zealand white of mixed sex, 6 to 8 weeks of age. These rabbits were fasted 24 hours before use to eliminate glycogen from the liver.

*Fractionation*—The animals were killed by decapitation and the tissues were rapidly removed to beakers containing ice-cold isotonic KCl and immersed in chopped ice, weighed, and homogenized in 9 volumes of isotonic (8.5 per cent) sucrose solution. The homogenates were fractionated exactly as previously described (5), with one exception. In the case of the tumor homogenates, the nuclear fraction was not washed because some of the nuclei failed to sediment when resuspended and centrifuged. Four frac-

<sup>1</sup> The authors wish to acknowledge the assistance of Mr. B. E. Kline in preparing the tumor transplants.

tions were obtained for each tissue: a nuclear fraction (N or N<sub>w</sub>), a mitochondrial fraction (M<sub>w</sub>), a submicroscopic particle fraction (P<sub>w</sub>), and a supernatant fluid (S<sub>2</sub>). The fractions were examined microscopically. The nuclear fraction contained large numbers of intact nuclei, some un-

TABLE II

*Total Nitrogen and Nucleic Acid Content of Fractions Obtained from Homogenates of Flezner-Jobling Rat Carcinomas*

Fraction	Nitrogen*		PNA phosphorus*			DNA phosphorus*		
	Total	A	Total	A	B	Total	A	B
	$\gamma$	<i>per cent</i>	$\gamma$	<i>per cent</i>	$\gamma$	$\gamma$	<i>per cent</i>	$\gamma$
Homogenate	1503	(100)†	55.2	(100)†	36.7	46.1	(100)†	30.6
N	559	37.2	20.9	37.8	37.4	39.4	85.4	70.7
M <sub>w</sub>	124	8.3	5.8	10.5	46.7			
P <sub>w</sub>	130	8.7	9.4	17.0	72.2			
S <sub>2</sub>	759	50.5	17.8	32.2	23.4			
Recovery.	1572	104.7	53.9	97.5		39.4	85.4	

\* Per 100 mg. of fresh tissue or its equivalent. Phosphorus calculated from pentose determinations (7). A, fraction of homogenate; B, per mg. of N.

† Figure assumed to be 100 per cent.

TABLE III

*Total Nitrogen and Nucleic Acid Content of Fractions Obtained from Homogenates of Rabbit Liver*

Fraction	Nitrogen*		PNA phosphorus*			DNA phosphorus*		
	Total	A	Total	A	B	Total	A	B
	$\gamma$	<i>per cent</i>	$\gamma$	<i>per cent</i>	$\gamma$	$\gamma$	<i>per cent</i>	$\gamma$
Homogenate	2580	(100)†	4.7	(100)†	18.1	22.4	(100)†	8.7
N <sub>w</sub>	574	22.2	13.1	28.0	22.8	22.3	99.5	38.9
M <sub>w</sub>	272	10.6	4.6	9.8	17.0			
P <sub>w</sub>	373	14.5	15.2	32.5	40.8			
S <sub>2</sub>	1260	48.9	14.8	31.6	11.8			
Recovery..	2479	96.2	47.7	101.9		22.3		

\* Per 100 mg. of fresh liver or its equivalent. Phosphorus calculated from pentose determinations (7). A and B, see Table I.

† Figure assumed to be 100 per cent.

broken cells, and no free mitochondria. Intact nuclei or nuclear fragments were not visible in any of the other fractions. The nitrogen and nucleic acid contents of the fractions are given in Tables II and III. Each figure represents the average of three separate experiments. Nitrogen was de-

TABLE IV  
*Flechner-Jobling Carcinoma*

Glycolysis obtained in 40 minutes with 30 mg. of tissue or fraction obtained therefrom.

Tissue fraction	Lactic acid produced per flask	Net P uptake per flask
	<i>micromoles</i>	<i>micromoles</i>
Homogenate.....	7.35	3.62
Nuclei (N).....	1.31	1.23
Mitochondria (M <sub>W</sub> ).....	0	-0.13
Microsomes (P <sub>W</sub> ).....	0.05	-0.76
Supernatant fluid (S <sub>2</sub> ).....	2.69	2.07
N+M <sub>W</sub> .....	1.99	1.19
N+P <sub>W</sub> .....	2.28	0.62
N+S <sub>2</sub> .....	4.23	2.42
M <sub>W</sub> +P <sub>W</sub> .....	0.38	-0.46
M <sub>W</sub> +S <sub>2</sub> .....	4.25	2.78
P <sub>W</sub> +S <sub>2</sub> .....	4.85	2.15
N+M <sub>W</sub> +P <sub>W</sub> +S <sub>2</sub> .....	7.28	3.33

TABLE V  
*Rabbit Liver*

Glycolysis obtained in 40 minutes with 30 mg. of tissue or fraction obtained therefrom.

Tissue fraction	Lactic acid produced per flask	Net P uptake per flask
	<i>micromoles</i>	<i>micromoles</i>
Homogenate.....	6.26	0.23
Nuclei (N <sub>W</sub> ).....	0.79	0.26
Mitochondria (M <sub>W</sub> ).....	0	0.05
Microsomes (P <sub>W</sub> ).....	0.17	-0.31
Supernatant fluid (S <sub>2</sub> ).....	3.30	0.42
N <sub>W</sub> +M <sub>W</sub> .....	0.84	0.49
N <sub>W</sub> +P <sub>W</sub> .....	1.74	-0.14
N <sub>W</sub> +S <sub>2</sub> .....	4.76	0.41
M <sub>W</sub> +P <sub>W</sub> .....	0.55	-0.15
M <sub>W</sub> +S <sub>2</sub> .....	4.30	0.07
P <sub>W</sub> +S <sub>2</sub> .....	5.94	0.27
N <sub>W</sub> +M <sub>W</sub> +P <sub>W</sub> +S <sub>2</sub> .....	6.55	0.20

terminated by a micro-Kjeldahl procedure (6) and nucleic acids by colorimetric reactions (7).

*Glycolytic Measurements*—These were made in Warburg respirometer flasks at 38° under anaerobic conditions in an atmosphere of 95 per cent

nitrogen-5 per cent carbon dioxide. The gassing of flasks and other manipulations were as described earlier (4). To each flask the following additions were made: 0.30 ml. of 0.024 M potassium phosphate (pH 7.4), 0.15 ml. of 0.5 M potassium bicarbonate, 0.30 ml. of 0.4 M nicotinamide, 0.15 ml. of 0.15 M potassium pyruvate, 0.10 ml. of 0.01 M adenosine triphosphate potassium salt, 0.20 ml. of 0.003 M diphosphopyridine nucleotide potassium salt, 0.20 ml. of 0.1 M magnesium chloride, 0.10 ml. of 0.3 M glucose, 0.15 ml. of 0.04 M hexose diphosphate potassium salt, 0.15 ml. of 0.2 M potassium fluoride, 0.30 ml. of water, isotonic sucrose (8.5 per cent) or tissue fraction in isotonic sucrose to make a total of 3.0 ml. Homogenate equivalent to 30 mg. of tissue (0.30 ml.) was used for each flask. The washed particle fractions were resuspended in isotonic sucrose to one-third the volume of the homogenate from which they were obtained and a corresponding amount used (0.10 ml. per flask). The supernatant fluid had necessarily a larger volume and correspondingly more of it was used per flask (0.60 ml.). The glycolysis measurements were made as soon as the fractionation was completed (5 hours). Carbon dioxide output was measured at 35° for 40 minutes; then 0.25 ml. of 65 per cent trichloroacetic acid was tipped in from the side arm of each flask to stop the reaction. The flask contents were analyzed for lactic acid and inorganic phosphorus by methods previously described (6). Data for carbon dioxide evolution are omitted in favor of direct analyses for lactic acid, since the latter are more specific.

Table IV gives the results of glycolysis measurements with Flexner-Jobling carcinoma homogenates, fractions, and recombinations. Table V gives the results obtained similarly for rabbit liver. Each figure in the tables represents the average of results from three separate experiments. Variations from one experiment to another were small.

#### DISCUSSION

The data for nucleic acid and nitrogen (Tables II and III) merit some comparison with the results obtained with other tissues (5, 8). The distribution of nitrogen in the fractions obtained from homogenates of Flexner-Jobling carcinoma was similar to that observed with liver tumors (8). The largest amounts of nitrogen were found in the nuclear fraction and supernatant, while the mitochondria and submicroscopic particle fractions contained very small amounts of nitrogen. Pentose nucleic acid (PNA) was present in all fractions but was more concentrated in the mitochondrial and submicroscopic particle fractions than in the homogenate. The reason for the poor recovery of desoxypentose nucleic acid (DNA) in the nuclear fraction of the Flexner-Jobling carcinoma (85.4 per cent) is not known, since microscopic examination of the fractions

indicated that nuclear material was present only in the nuclear fraction. A comparison of the results obtained with rabbit liver (Table III) with those previously reported for rat liver (5) shows that the most striking difference between the two tissues was the greatly decreased amounts of nitrogen recovered in the mitochondrial and submicroscopic particle fractions of the former. As in the case of rat liver, PNA was more concentrated only in the submicroscopic particle fraction and the entire DNA of the rabbit liver homogenate was recovered in the nuclear fraction.

The glycolysis data can be examined with regard to which fractions of the cell are able to glycolyze hexose diphosphate (*i.e.*, form lactic acid) and which are able to give phosphorus uptake. The interpretation is admittedly complicated by the possibility that lactate formation may be limited by lack of any of the enzymes concerned with the phosphate acceptor-transmitter system. When these are absent from a fraction, its glycolysis might cease owing to lack of a phosphate acceptor. It is known that adenosinetriphosphatase tends to be associated with the particulate matter (3, 8).

The results for tumor and normal liver are quite similar with regard to distribution of glycolytic enzymes. Liver does not give the strong phosphorylation reaction that is obtained with tumor under these conditions. It appears that the main glycolytic activity is in the soluble fraction ( $S_2$ ). However, it is not possible to get a rate approaching that of the homogenate without the addition to the soluble enzymes of one of the particulate fractions. Since the phosphorus uptakes by the soluble fraction are quite efficient, in relation to the amount of glycolysis, without the addition of particulate fractions, it does not seem likely that the hexokinase is lacking from the soluble fraction. It therefore appears that the particulate matter is needed mainly to add adenosinetriphosphatase in order that phosphate acceptor may be made more rapidly available. It is obvious that no fraction or pair of fractions can give the full activity of the whole homogenate, though when all fractions are recombined, the original activity is achieved. In the tumors, no single fraction gave as high an activity per unit of protein nitrogen as the original homogenate.

Some glycolytic activity is shown by the nuclear fractions. This is probably due to the presence of some whole cells, and in the case of the tumor to the presence of impurities from the other fractions, since the tumor nuclei were not washed.

#### SUMMARY

Homogenates of Flexner-Jobling rat carcinoma and rabbit liver were separated centrifugally to give four definite fractions: (a) a nuclear fraction, (b) a mitochondrial fraction, (c) a submicroscopic particle fraction, (d) a

"soluble" fraction. Measurements were made of lactic acid production and net phosphorus uptake in an optimal medium under anaerobic conditions. The glycolytic enzymes appear to be in the "soluble" fraction, though addition of any of the particulate fractions markedly increases the rate. No single fraction or pair of fractions is able to reach the activity of the original homogenate, though this is achieved when all fractions are recombined. It is concluded that in any study of glycolytic enzymes no fraction of the cell should be used without making a study of the relationship between it and the total cell contents.

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# AN INVESTIGATION OF THE BIOLOGICAL BEHAVIOR OF THE SULFUR ANALOGUE OF CHOLINE\*

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(Received for publication, July 16, 1948)

During the course of investigations on transmethylation involving compounds related to choline (1, 2), it was found that betaine (3) and also its sulfur analogue, dimethylthetin (4), supported growth of the white rat on a choline-methionine-free diet containing homocystine, signifying that these compounds were able to supply essential methyl groups to the body. Dimethylthetin (sulfobetaine) was of particular interest because of its being a methylsulfonium compound. In view of its remarkable growth-promoting properties when administered in a methyl-free diet to rats, the studies have been extended to include the corresponding sulfur analogue of choline,  $\beta$ -hydroxyethyltrimethylsulfonium chloride, which we have termed for convenience "sulfocholine chloride."

## EXPERIMENTAL

*Preparation of Sulfocholine Chloride*—Renshaw, Bacon, and Roblyer (5) described the preparation of  $\beta$ -hydroxyethyltrimethylsulfonium iodide by the interaction of ethylene iodohydrin and dimethyl sulfide maintained at room temperature for several days. These workers noted that this method of preparation can result in the formation of considerable amounts of the trimethylsulfonium salt, due to the dissociation of the  $\beta$ -hydroxyethyltrimethylsulfonium iodide, formed in solution, to methyl iodide and the subsequent reaction between the methyl iodide and the dimethyl sulfide present. Other non-crystallizable, oily products were also formed in this reaction. We have repeated this method of synthesis and have obtained similar results. The unavoidable formation of trimethylsulfonium salts during the preparation of methylsulfonium compounds, when dimethyl sulfide and an alkyl halide are used as starting materials, has been reported by other workers (6). We therefore describe an improved synthesis of sulfocholine iodide which obviates any formation of trimethylsulfonium iodide as a contaminant. Ethylene bromohydrin is converted to methyl  $\beta$ -hydroxyethyl

\* The authors wish to express their appreciation to the Commonwealth Fund and to the Lederle Laboratories Division, American Cyanamid Company, for research grants which have aided greatly in this work.

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sulfide and the latter is treated with methyl iodide to give sulfocholine iodide in quantitative yield.

Ethylene bromohydrin (62 gm.) was added slowly to methyl mercaptan (25 gm.) dissolved in sodium methoxide (12 gm. of sodium in 200 cc. of absolute methanol). The formation of methyl  $\beta$ -hydroxyethyl sulfide was immediate and was completed by refluxing the product for 30 minutes. The supernatant liquid was fractionated under reduced pressure. The thio ether distilled as a colorless oil, b.p. 74–75° at 22 mm., and was halogen-free. Methyl  $\beta$ -hydroxyethyl sulfide (9.2 gm.) was added to methyl iodide (14.2 gm.) in a tightly stoppered bottle. The formation of the sulfonium iodide commenced within 10 minutes with the evolution of much heat and the whole reaction mixture had set to an orange crystalline mass within a further 20 minutes. The iodide was converted to the chloride without preliminary purification by shaking in aqueous solution with fresh silver chloride. On removal of the silver iodide formed, the aqueous solution was evaporated to dryness under reduced pressure and dried at 40° by means of an oil pump. The product was obtained as a slightly yellow viscous oil which crystallized to a highly deliquescent solid on being left overnight in the refrigerator.

$C_4H_{11}SOCl$ .	Calculated.	C 33.7, H 7.8, S 22.5, Cl 24.9
	Found.	" 33.8, " 7.8, " 22.3, " 24.7

Sulfocholine chloroplatinate crystallized as orange needles from aqueous ethanol, capillary m.p. 190° (corrected).

*Feeding Experiments*—Young male albino rats of the Rockland strain were used. The animals were placed for 1 week on a preliminary diet of the following percentage composition: casein 20, fat (Covo) 19, Mazola corn oil 1, salt mixture (7) 4, sucrose 55.6, fat-soluble and water-soluble vitamins (4), L-cystine 0.4. The animals were then transferred to the experimental diet of the following composition: amino acid mixture (1)<sup>1</sup> 18.5, fat (Covo) 19, Mazola corn oil 1, salt mixture (7) 4, fat-soluble and water-soluble vitamins (4), DL-homocystine 0.87, L-cystine 0.4, the compound under test and sucrose to make up to 100 per cent. Sulfocholine chloride was initially fed at a level of 0.77 per cent, corresponding on the basis of methyl groups to 0.5 per cent choline chloride. In subsequent experiments lower dietary levels were also used. Total liver lipides were determined by the method of Best, Channon, and Ridout (8).

<sup>1</sup> In the amino acid mixture used, a level of 1.9 per cent L-lysine hydrochloride, together with an equivalent amount of sodium bicarbonate (0.9 per cent), was employed, and the dietary level of the mixture was 18.5 per cent.

## Results

*Growth Studies*—Preliminary growth studies carried out on rats fed 0.77 per cent sulfocholine chloride in a methionine-choline-free diet containing homocystine showed that this compound, unlike dimethylthetin, was not able to support growth. Furthermore, it was toxic and resulted in the death of the animals in 2 to 3 weeks. Growth experiments were therefore extended to lower dietary levels and the results obtained are given in Table I and compared with those for rats on the methyl-free diet with and without

TABLE I

*Growth Rates of Rats on Methyl-Free Diets Supplemented with Sulfocholine Chloride and Methyl  $\beta$ -Hydroxyethyl Sulfide*

Compound under test	Rat No.	Days on diet	Weight change	Food intake	Growth rate	Condition of kidneys
			gm.	gm. per day	gm. per day	
Basal methyl-free diet	50	12*	81-71	3.4	-0.8	Hemorrhagic
	25	21	113-95	5.0	-0.9	"
	28	21	97-89	4.8	-0.4	"
Sulfocholine chloride 0.2%	129	32	78-89	5.2	+0.3	"
	121	32	75-85	5.3	+0.3	"
Sulfocholine chloride 0.39%	122	19*	92-54	4.7	-2.0	Normal
	133	23	106-107	4.6	0.0	"
Sulfocholine chloride 0.77%	44	16*	86-59	4.9	-1.7	"
	55	15*	93-65	3.9	-1.9	"
	52	21*†	105-71	5.6	-1.6	"
Methyl $\beta$ -hydroxyethyl sulfide 0.99%	43	12*	101-72	2.9	-2.4	Hemorrhagic
	60	11*	89-74	3.2	-1.4	"
Choline chloride 0.5%	49	23	95-141	8.3	+2.0	Normal
	51	21	85-153	9.2	+3.2	"
	24	21	108-165	8.8	+2.7	"

\* Rat died.

† Level of compound in diet reduced to 0.39 per cent after 8 days.

added choline. At all three levels administered, sulfocholine was unable to promote growth. However, at the 0.2 per cent level there was no evidence of toxicity and over a period of 32 days the rats maintained their body weights and even gained slightly. Some maintenance of body weight was also observed in the case of other rats at the higher dietary levels over an initial period of about 1 week and before the toxic effects of the compound had become evident. This was in contrast to the behavior of the animals on the methyl-free diet. These rats suffered consistent weight losses immediately after they were placed on the experimental diet. Considering the rats on the three dietary levels as one group, it was observed that twelve of

the nineteen animals under investigation maintained their body weight to within  $\pm 5$  gm. over the first 7 days, four lost more than 5 gm., and three gained more than 5 gm. A number of the animals showed weight maintenance over longer periods than 7 days. The possible significance of this observation is discussed in a further section.

On the basis of previous studies with choline (9) and betaine (10), the inability of the sulfur analogue of choline to support the growth of rats on choline-methionine-free diets supplemented with homocystine suggests that sulfocholine cannot supply labile methyl groups for transmethylation processes. This result is somewhat surprising in view of the fact that sulfocholine may be considered as structurally intermediate between the two highly active methyl donors, choline and dimethylthetin, and may have



been expected on this account to show some activity. It clearly emphasizes the marked structural specificity associated with the ability of a compound to act as a methyl donor (2). It is of interest that Dubnoff and Borsook (11) have reported the existence in rat and guinea pig liver of three trans-methylases specifically concerned with the methylation of homocysteine by choline, betaine, and dimethylthetin, respectively.

At levels of 0.39 per cent and above in the diet, sulfocholine proved toxic to rats and resulted in their death within 2 to 3 weeks. The symptoms of toxicity were not manifest until about 1 week after the administration of the compound, although some rats during this period seemed more nervous and excitable than control animals. On about the 8th to the 10th day a black speck appeared in the corner of each eye in the neighborhood of the tear duct. This developed into an acute exudative inflammation of the anterior palpebral fissure of each eye. During the next 2 days this spread posteriorly along the conjunctival cutaneous junction, involving the rims of both eyelids and resulting in closure of the eyes. At this stage the corners of the mouth and occasionally the anus were found to be inflamed and encrusted and the skin of the forepaws and chest became red and inflamed. On autopsy no general gross pathology was observed, although in some rats the kidneys appeared slightly enlarged.

These toxic symptoms did not appear in rats fed 0.99 per cent methyl  $\beta$ -hydroxyethyl sulfide along with the methyl-free diet. However, as indicated in Table I, these animals failed to grow and died within 2 weeks, as did one of the animals on the basal diet. They were found on autopsy to have markedly hemorrhagic kidneys. This renal damage due to a methyl deficiency seems most likely to have been the cause of death. Another animal which survived on the experimental diet for 43 days likewise did not show the eye or mouth lesions described.

In view of the possibility of some of the symptoms being due to an irritant action of the compound as a result of contact with the diet, several rats were treated daily for 3 weeks with a 1 per cent aqueous solution of sulfocholine on a shaved patch of skin between the ears. No observable effect was obtained by this treatment.

The actual mechanism of the toxicity is not yet understood, but a number of possible explanations have been examined. It was considered that sulfocholine might be acting as a choline antagonist, despite the fact that the symptoms of toxicity do not resemble those of a simple choline deficiency. Rats were maintained for 6 days on the preliminary 20 per cent casein diet and were then transferred to the amino acid diet containing homocystine and supplemented by sulfocholine and choline in the following ways: 0.77 per cent sulfocholine as the chloride was fed for 10 days and 0.5 per cent choline was then added; 0.5 per cent choline was fed along with the 0.77 per cent sulfocholine for 10 days and the choline level was then raised to 1 per cent; 0.5 per cent choline was fed for 10 days before the administration of 0.77 per cent sulfocholine, and after another 10 days the choline level was raised to 1 per cent. All rats, whether given choline some days after, simultaneously with, or even prior to the addition of sulfocholine to the diet, died within 14 to 16 days.

Dimethylthetin chloride was also unable to prevent the eye and mouth lesions resulting from the feeding of sulfocholine. Rats transferred from the methyl-free diet containing 0.84 per cent dimethylthetin chloride to the same diet with added 0.6 per cent sulfocholine developed these symptoms after a period of about 10 days. Rats maintained on the preliminary 20 per cent casein diet supplemented with 0.77 per cent sulfocholine also developed the toxic symptoms, lost weight, and died within 2 weeks.

Since the inflammation of the corners of the mouth and eyes caused by sulfocholine is suggestive of a riboflavin deficiency, two rats just showing the toxic symptoms on the methyl-free diet containing 0.77 per cent sulfocholine were given 200  $\gamma$  of riboflavin per day orally for 9 days. No alleviation of the condition of the eyes and mouth was observed. This would suggest that sulfocholine administration was not producing a riboflavin deficiency.

*Lipotropic and Kidney Antihemorrhagic Properties*—It was observed, as pointed out earlier, that, when first placed on methyl-free diets containing sulfocholine, rats did not generally suffer marked weight losses for the 1st week and in some cases for a longer period. This finding was interpreted as indicating that sulfocholine might be replacing choline in at least some of its metabolic rôles, so making available a small amount of tissue choline for transmethylation. This idea was strengthened by the finding, indicated in Tables I and II, that the kidneys of rats fed 0.39 per cent and 0.77 per

cent sulfocholine were definitely protected against hemorrhagic damage. Subsequent experiments have shown that not one of the seventeen animals fed sulfocholine at these two levels developed hemorrhagic kidneys. Of seven animals fed 0.2 per cent sulfocholine, four developed hemorrhagic kidneys and three were protected. Determinations of total liver lipides were carried out on rats fed the compound at the three dietary levels, 0.2 per cent, 0.39 per cent, and 0.77 per cent, for a 10 day period. The results are shown in Table II and compared with those for rats on comparable food intakes of a methyl-free diet. Two rats given the methyl-free diet containing added choline for the same period gave total liver lipide values of 3.0 and 3.7 respectively. The results clearly demonstrate that sulfocholine

TABLE II

*Total Lipides of Livers of Rats Fed Sulfocholine at Various Levels for 10 Day Period*

Level of sulfocholine	Rat No.	Food intake	Weight change	Liver lipides	Condition of kidneys
<i>per cent</i>		<i>gm</i>	<i>gm.</i>	<i>per cent wet weight liver</i>	
0 (Methyl- free diet)	265	48	-7	12.7	Hemorrhagic
	271	49	-12	12.9	"
	277	43	-12	9.8	"
0.2	340	60	+18	6.8	Normal
	336	45	-5	8.3	"
	328	50	-3	18.9	Hemorrhagic
0.39	276	59	0	4.8	Normal
	280	54	+3	5.2	"
	279	50	+2	3.0	"
0.77	263	46	-4	3.6	"
	278	46	-7	5.1	"
	274	45	-7	5.4	"

is actively lipotropic at dietary levels of 0.39 per cent and above. At a level of 0.2 per cent, the lipotropic activity was only partially evident.

It might be pointed out that the protection against fatty livers and hemorrhagic kidneys was not the result of a low food intake, since the protected animals ate an amount equivalent to that consumed by the control animals.

It has been established that the lipotropic properties of arsenocholine (12, 13) and triethylcholine (14) are due to the ability of these compounds partly to replace choline in the liver phospholipides. It seemed likely that sulfocholine might be lipotropic for the same reason and an attempt was therefore made to detect sulfocholine in the liver fat of animals fed this compound. The livers were first homogenized and extracted with hot ethanol. This process would be expected to remove any unbound

sulfocholine present, as well as the total lipides. The residue obtained upon evaporation of the ethanol was then extracted with hot absolute ether, thus separating the lipides from any insoluble free sulfocholine. Upon removal of the solvent, the individual liver fats were pooled in groups of three and heated in sealed tubes with excess sodium ethoxide at 70° for 3 days. Under these conditions we have found sulfocholine to be decomposed to dimethyl sulfide, as in the case of other alkyl dimethylsulfonium salts (15). The contents of the tubes were then examined for dimethyl sulfide by breaking each tube in a stout bottle connected to a chain of bubbler tubes containing saturated mercuric chloride solution. Air was drawn through the apparatus, whereupon a voluminous white precipitate was formed in the first mercuric chloride trap. This mercuric chloride complex was recrystallized from an acetone-benzene mixture. The melting point of the compound (151° (corrected)) agreed with that of the corresponding mercuric chloride complex prepared from pure dimethyl sulfide, as did the melting point of a mixture of the two substances. Analyses for sulfur and chlorine were made on the mercuric chloride derivative of the sulfide obtained from the liver fats and were compared with those for authentic dimethyl sulfide. The mercuric chloride derivative of dimethyl sulfide possessed a sulfur content of 6.45 per cent and a chlorine content of 23.2 per cent. The mercuric chloride derivative of the sulfide from the liver fats possessed a sulfur content of 6.26 per cent and a chlorine content of 23.0 per cent. The volatile sulfide thus appears to be dimethyl sulfide. Its isolation from liver fats so treated with sodium ethoxide affords evidence that sulfocholine was present in combined form in the phospholipides. This is in accord with the deductions from the analytical data in Table II and establishes the lipotropic nature of sulfocholine.

The authors wish to thank Miss Josephine E. Tietzman for performing the microanalyses and Mrs. Audrey Kellogg Hafford for technical assistance in connection with this problem.

#### SUMMARY

An improved synthesis of  $\beta$ -hydroxyethyldimethylsulfonium iodide and its conversion to the corresponding chloride are described. This latter compound, referred to as "sulfocholine chloride," has been found to be incapable of supporting the growth of rats on diets free of choline and methionine and containing homocystine. The compound was toxic above a level of 0.2 per cent in the diet. Sulfocholine has been found to be active in preventing the development of fatty livers and renal hemorrhages in rats fed the methyl-free diet.

A volatile sulfide has been isolated from the livers of such rats by a



procedure which is known to cause the degradation of sulfocholine to dimethyl sulfide. The sulfide has been identified as dimethyl sulfide. This is presented as evidence that the lipotropic activity of sulfocholine is due to its incorporation into liver phospholipides in place of choline.

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# COMPOUNDS RELATED TO DIMETHYLTHETIN AS SOURCES OF LABILE METHYL GROUPS\*

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(Received for publication, August 3, 1948)

Previous studies carried out in this laboratory (1-4) have shown that the only compounds so far known to be capable of supporting the growth of the rat on a choline-methionine-free diet supplemented with homocystine are choline itself (and choline derivatives, *e.g.* lecithin and phosphorylcholine), dimethylethyl- $\beta$ -hydroxyethylammonium chloride (monoethylcholine), betaine, and dimethylthetin (sulfobetaine). On the basis of experiments involving the feeding of deuteriocholine and deuteriobetaine to rats (5, 6), the activity of compounds in supporting growth under these conditions is interpreted as signifying that they are able to transfer methyl groups to homocystine to form methionine. In addition to promoting growth, these methyl-donating compounds are also able to prevent the formation of fatty livers (3). However, other compounds devoid of available methyl groups are active lipotropic agents, and thus the ability to act as a lipotropic agent cannot be considered synonymous with the ability to act as a donor of essential methyl groups (7).

In view of the pronounced methyl-donating activity of the sulfur analogue of betaine (4) and the lack of this activity in the case of the corresponding sulfur analogue of choline (8), it became of interest to extend these investigations to other sulfonium compounds in order to gain further information of the structural specificity involved. Compounds tested included methylethylthetin, diethylthetin, and dimethyl- $\beta$ -propiothetin. The last named compound appeared of special significance, since it has recently been isolated from a marine alga, *Polysiphonia fastigiata*, by Challenger and Simpson (9). In a preliminary communication we have already reported it to be an excellent substitute for choline in methyl-free diets containing homocystine (10).

## EXPERIMENTAL

*Preparation of Compounds*—S-Methylthioglycolic acid was prepared by methylation of ethyl thioglycolate with methyl iodide in alcoholic sodium

\* The authors wish to express their appreciation to the Commonwealth Fund and to the Lederle Laboratories Division, American Cyanamid Company, for research grants which have aided greatly in this work.

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methoxide and hydrolysis of the resulting ester with sodium hydroxide. Dimethylthetin, methylethylthetin, and diethylthetin were obtained as their chlorides by the interaction of monochloroacetic acid with dimethyl, methylethyl, and diethyl sulfides, respectively, in nitromethane as solvent. Dimethyl- $\beta$ -propiothetin chloride was prepared by treatment of  $\beta$ -iodopropionic acid with dimethyl sulfide in nitromethane and conversion of the resulting sulfonium iodide to the chloride by shaking with dry silver chloride in absolute ethanol.

*Feeding Experiments*—Young male albino rats of the Rockland strain were used. The animals were placed on a preliminary diet containing 20 per cent casein (Diet I, composition given in the following section). After being kept on this diet for approximately 1 week they were transferred for 7 days to a diet containing pure amino acids as the protein component (1)<sup>1</sup> together with 1 per cent DL-methionine (Diet II). The rats showing most satisfactory growth at this stage (a growth rate of 2 to 3 gm. per day) were then selected for the feeding of the compounds under investigation. The animals were transferred to a diet similar to Diet II, with the exception that the methionine was replaced by 0.87 per cent DL-homocystine together with the compound under test (Diet III).

*Composition of Diets*—The percentage composition of the diets was as follows: Diet I, casein 20, fat (Covo) 19, sucrose 55.6, salt mixture (11) 4, Mazola corn oil 1, fat-soluble and water-soluble vitamins as reported in a previous publication (4), L-cystine 0.4. Diet II, amino acid mixture (1)<sup>1</sup> 18.5, sucrose 56.1, DL-methionine 1.0, L-cystine 0.4, fat, salt mixture, and vitamins as in Diet I. Diet III, amino acid mixture (1)<sup>1</sup> 18.5, DL-homocystine 0.87, L-cystine 0.4, fat, salt mixture, and vitamins as in Diet I, the compound under test and sucrose to make up to 100 per cent. Methylthioglycolic acid, dimethylthetin chloride, and dimethylpropiothetin chloride were administered in amounts corresponding on the basis of methyl groups to an arbitrarily chosen level of 0.5 per cent choline chloride. Methylethylthetin chloride and diethylthetin chloride were given in amounts equivalent on a molar basis to the dimethylthetin chloride fed.

*Preparation of S-Trideuteriomethylthioglycolic Acid*—Ethyl thioglycolate (2.4 gm.) was dissolved in sodium methoxide (0.46 gm. of sodium in 7 cc. of absolute methanol) at  $-10^{\circ}$ , and 3.0 gm. of trideuteriomethyl iodide (12) were added gradually. The resulting solution gave a negative test for the sulfhydryl grouping. The methanol was evaporated, the residue was heated with 3 cc. of water, and the ethyl deuteriomethylthioglycolate was saponified by the dropwise addition of sufficient 15 per cent potassium

<sup>1</sup> In the amino acid mixture used, a level of 1.9 per cent of L-lysine hydrochloride, together with an equivalent amount of sodium bicarbonate (0.9 per cent), was employed, and the dietary level of the mixture was 18.5 per cent.

hydroxide to keep the reaction mixture alkaline to brom-thymol blue. The solution was then acidified with concentrated hydrochloric acid and the trideuteriomethylthioglycolic acid was extracted with two 7 cc. portions of ether, dried over sodium sulfate, and distilled at 112–113° at a pressure of 12 mm.

*Analyses*—Trideuteriomethylthioglycolic acid

Elementary.<sup>2</sup> Calculated, S 29.77; found, S 29.71

Deuterium.  $53.1 \pm 0.7$  atom % excess in methyl group

*Feeding Experiment*—Two S5 gm. rats were maintained on the preliminary diet (Diet I) for 6 days and then were transferred for 11 days to Diet III together with 1.14 per cent S-trideuteriomethylthioglycolic acid and 0.9 per cent sodium bicarbonate to neutralize the acid. (The addition of the base was effective in reducing almost completely the odor of the compound in the diet.) Food intakes were determined daily and further supplements of the sodium salt of trideuteriomethylthioglycolic acid in water were given by stomach tube in order to make up the daily intake to 70 mg. or to that amount which would be supplied in 6 gm. of diet. Since the food intakes were 4 to 5 gm. per day, the amount administered by stomach tube represented only a small fraction of the total dose. At the end of the period the rats were sacrificed. Carcass choline was isolated as the chloroplatinate, creatine as creatinine potassium picrate (12), and both compounds were analyzed for deuterium (13).

### Results

In Table I are shown the growth rates and food intakes of rats fed the various compounds under discussion in methyl-free diets containing homocystine. For comparison the corresponding data for rats on a methyl-free diet with and without the choline supplement are given. Dimethylthetin and dimethylpropiothetin were clearly able to support growth as well as choline itself and produced no apparent toxic effects at the dietary levels used. The rats maintained on these compounds remained in excellent health throughout the experiment and in no case was there a detectable growth lag during the transfer from the methionine-containing diet to the diet containing homocystine and the compound under test. Methylethylthetin appeared to be somewhat less active as a methyl donor. Two of the rats on this compound grew as well as those on dimethylthetin; four animals grew at a slower rate although they fared substantially better than those on the methyl-free diet; one died after 10 days. Diethylthetin was inactive in supporting growth.

<sup>2</sup> The calculated value is based on the increased molecular weight due to deuterium in the molecule.

Both dimethylthetin and dimethylpropiothetin prevented the formation of hemorrhagic kidneys in the animals studied. Methylene-thetin definitely prevented hemorrhagic kidneys in five out of the seven animals.

TABLE I  
*Growth Rates and Food Intakes of Rats Fed Dimethylthetin and Related Compounds*

Compound under test	Rat No.	Days on diet	Weight change	Food intake	Growth rate	Condition of kidney
			gm.	gm. per day	gm. per day	
Basal methyl-free diet	3650	27	96-69	4.1	-1.0	Hemorrhagic
	3654	27	99-89	4.6	-0.4	"
	3660	10*	103-86	4.3	-1.7	"
	50	12*	81-71	3.4	-0.8	"
	25	21	113-95	5.0	-0.9	"
	28	21	97-89	4.8	-0.4	"
Choline 0.5%	3643	23	93-139	8.6	+2.0	Normal
	49	23	95-141	8.3	+2.0	"
	51	21	85-153	9.2	+3.2	"
	24	21	108-165	8.8	+2.7	"
Dimethylthetin 0.84%	3644	24	90-150	7.4	+2.5	"
	3658	24	107-186	9.2	+3.3	"
	3661	27	78-124	6.6	+1.7	"
Dimethylpropiothetin 0.92%	41	21	98-158	8.8	+2.9	"
	48	21	88-137	7.9	+2.3	"
	57	21	101-180	9.3	+3.8	"
Methylene-thetin 0.92%	3649	16	88-94	5.7	+0.4	"
	3651	10*†	103-71	4.0	-3.2	"
	3653	16	83-104	6.9	+1.3	"
	3656	23	89-145	9.4	+2.4	"
	47	21	84-93	5.1	+0.4	?
	54	23	96-136	6.7	+1.7	Hemorrhagic
Diethylthetin 0.99%	26	21	109-163	8.1	+2.6	Normal
	3645	27	85-61	3.5	-0.9	Hemorrhagic
	3655	27	93-79	5.2	-0.5	"
	3657	27	105-84	4.9	-0.8	"
S-Methylthioglycolic acid 0.5%	3646	13*	89-75	3.5	-1.1	"
	3659	27	104-93	5.2	-0.4	"
S-Methylthioglycolic acid 1.14%	3648	14*	103-58	3.4	-3.2	"
	3652	27	85-63	3.5	-0.8	"

\* Rat died.

† Cause of death unknown.

In another rat of this group, protection was doubtful. Diethylthetin was completely ineffective.

Dimethylthetin has previously been reported as being lipotropic by Welch, as referred to by Moyer and du Vigneaud (3). In Table II are

shown parallel data obtained from rats given methylethylthetin and dimethylpropiothetin. The liver fat values are compared with the corresponding figures for rats on a methyl-free diet with and without added choline. Dimethylpropiothetin was highly active as a lipotropic agent, as would be expected from its ability to promote growth on homocystine diets

TABLE II  
*Total Liver Lipide Values*

Compound under test	Rat No.	Days on diet	Food intake	Total liver lipides
			gm. per day	per cent wet weight liver
Basal methyl-free diet	25	21	5.0	16.6
	28	21	4.8	20.6
Choline 0.5%	3643	23	8.6	4.7
	24	21	8.8	4.8
	51	21	9.2	4.1
Dimethylpropiothetin 0.92%	57	21	9.3	5.2
	41	21	8.8	5.2
	48	21	7.9	4.0
Methylethylthetin 0.92%	3649	16	5.7	4.4
	3653	16	6.9	5.3
	3656	23	9.4	5.5
	47	21	5.1	12.5
	54	23	6.7	5.6
	26	21	8.1	10.9

TABLE III  
*Feeding Experiment with S-Trideuteriomethylthioglycolic Acid\**

Rat No.	Change in body weight	Choline <sup>†</sup> isolated		Creatine isolated	
		Deuterium in methyl groups	Per cent derived from compound fed	Deuterium in methyl groups	Per cent derived from compound fed
39	102-92	0.49 ± 0.25	0.91	0.52 ± 0.40	0.99
21	99-88	0.52 ± 0.13	0.99	0.37 ± 0.39	0.70

\* The experiments were conducted over an 11 day period.

† Analyses for choline chloroplatinate: Rat 39, calculated, Pt 31.6, found, 31.8; Rat 21, calculated, Pt 31.6, found, 32.2 per cent.

and to prevent renal hemorrhages. Methylethylthetin was again not as active as dimethylthetin, the former compound protecting four out of the six rats from fatty livers.

As shown in Table I, S-methylthioglycolic acid, the sulfur counterpart of dimethylglycine, was unable to support growth at the two levels fed. The

higher level corresponds on the basis of methyl groups to 0.5 per cent choline, but since it was considered that the compound might be toxic at such a concentration a lower level was also used. The compound was also unable to prevent the onset of hemorrhagic kidneys.

In view of the close structural relationship of S-methylthioglycolic acid to dimethylthetin, it seemed possible that methylthioglycolic acid might be capable of acting as a precursor of dimethylthetin in the body, thus acting as an indirect methyl donor. It may be recalled that dimethylaminoethanol (14) has been shown to act as an indirect methyl donor, being unable to promote growth appreciably, yet able to furnish methyl groups which eventually find their way into tissue creatine and choline.

To investigate the rôle of methylthioglycolic acid, this compound was prepared with its methyl group labeled with deuterium and was fed to two rats. After a period sufficiently long for detectable transmethylation to have taken place, the rats were killed and the tissue choline and creatine were analyzed for deuterium. The results are shown in Table III. Less than 1 per cent of the deuteriomethyl groups was found to be present in the choline and creatine and in the case of the latter compound the amount present was within the range of experimental error. The extent of this methyl transfer is comparable to that obtained from sarcosine (15) and dimethylglycine (6), both regarded as poor methyl donors. These results indicate that methylthioglycolic acid is not an active source of methyl groups. In addition, the results suggest that the methylation of methylthioglycolic acid to dimethylthetin does not take place to an appreciable extent in the body under the conditions described.

#### DISCUSSION

The demonstration by du Vigneaud, Moyer, and Chandler of the ability of dimethylthetin to promote the growth of rats on methyl-free diets (4), together with the present findings of the similar activity of dimethylpropiothetin (10), clearly marks these methylsulfonium compounds as a new class of methyl donors. These *in vivo* experiments are in agreement with the independent *in vitro* experiments of Dubnoff and Borsook (16), who have shown dimethylthetin<sup>3</sup> to be an extremely active methyl donor for homocysteine in liver homogenates. The earlier observation of Welch (3) that dimethylthetin is a lipotropic agent and our data that methylethylthetin and dimethylpropiothetin are also lipotropic add further support. In addition, Welch<sup>4</sup> has reported that dimethylthetin and methylethylthetin

<sup>3</sup> In the oral presentation of their paper on dimethylthetin (meeting of the American Society of Biological Chemists at Atlantic City, March 15, 1948), the abstract of which is referred to, Dubnoff and Borsook reported that dimethylpropiothetin was also active in promoting methionine synthesis.

<sup>4</sup> Welch, A. D., private communication.

are able to protect animals on methyl-free diets from renal hemorrhagic damage. Our results are in confirmation and also include dimethylpropiothetin as an antihemorrhagic agent.

The position of methylethylthetin as a methyl donor appears to be a border line one and intermediate between dimethylthetin and diethylthetin, since it was capable of supporting good growth in only two out of the seven animals under test. The compound was able to protect all but two of the animals from fatty livers and all but two from renal hemorrhagic damage. Its lower activity compared with dimethylthetin may possibly be associated with the presence in the molecule of an ethyl group bound in a similar manner to the methyl group. It has previously been observed that the replacement of the methyl groups in choline and methionine by ethyl groups leads to decreased growth-promoting activity and increased toxicity (3, 17).

Diethylthetin was unable to support growth and animals fed on this compound developed hemorrhagic kidneys. Welch has found it to be inactive as a lipotropic agent.<sup>4</sup> Triethylcholine, also devoid of methyl groups, is known, however, to be a lipotropic and antihemorrhagic agent on account of its ability to replace the choline molecule as a whole in certain of its metabolic rôles (3, 18). Diethylthetin must therefore be unable to replace the entire choline molecule or to form a further compound in the body which is effective. In this respect it resembles arsenobetaine and phosphobetaine (19).

The original discovery by Challenger and Simpson of the presence of dimethylpropiothetin in *Polysiphonia fastigiata* (9), which prompted growth studies on this compound, introduces the possibility that substances of this type may be present in other organisms. Moreover, the fact that dimethylthetin and its homologue have been found to be active methyl donors in the rat coupled with the report of Dubnoff and Borsook (16) that an enzyme specifically catalyzing the methylation of homocysteine by dimethylthetin exists in rat and guinea pig liver tissue would suggest that either dimethylthetin or a homologue or closely related derivative may actually be a tissue constituent and may take part in normal methylation processes. If this is the case, such compounds would have to be considered as potential dietary factors, and a diet deficient in methionine, choline, and betaine alone could no longer be considered as methyl-free unless methyl-donating thetins had been shown to be absent.

Many of the earlier studies on the metabolism of choline, betaine, and related compounds have been directed towards modification of the structure of the compound in question in order to determine to what extent the molecular configuration may be altered without effecting a loss in biological activity. With lipotropism as the biological activity in mind, it has been possible, in the case of choline, to make considerable structural changes,



including complete replacement of methyl by ethyl groups (18), and replacement of the nitrogen atom by phosphorus (19), arsenic (20), and sulfur (8) with retention of the lipotropic properties. The most reasonable explanation at the present time seems to be that the various substituted cholines are being utilized in the liver for phospholipide formation in lieu of choline itself and are active merely by virtue of being choline analogues.

In the case of methyl-donating ability, if the assumption is made that dimethylthetin is active because it is the sulfur analogue of betaine, then one might expect sulfocholine to be at least as effective, if not more so. However, it has been shown not to be a methyl donor and to be toxic above a certain dietary level (8). Furthermore, whereas dimethylpropiothetin exhibits a marked growth-promoting activity, its nitrogen analogue,  $\beta$ -alanine betaine, is toxic and apparently not a methyl donor (3). The concept of the methylsulfonium compounds under discussion as being methyl donors simply on account of their structural similarity to nitrogen compounds already known to take part in transmethylation reactions would seem misleading, particularly in view of the existence of a separate enzyme system concerned with transmethylation from these compounds (16). The thetins referred to must be considered as methyl donors in their own right and not because of their analogy to betaine.

From an examination of the structures of the six compounds which have been shown to be capable of methylating homocystine *in vivo*, namely choline, monoethylcholine, betaine, dimethylthetin, methylethylthetin, and dimethylpropiothetin, it appears that the structural criterion associated with this process is the presence, in the molecule of the potential methyl donor, of a methyl group or groups directly attached to an onium pole. Growth-promoting activity is not exhibited by compounds lacking either a methyl group or an onium structure, although lipotropic properties may still be exhibited by such compounds. On the one hand, triethylcholine (1, 3) and diethylthetin, lacking available methyl groups, are inactive in promoting growth. On the other hand, N-methyl and S-methyl compounds such as methylaminoethanol and dimethylaminoethanol (14), sarcosine (15), dimethylglycine (6), methyl  $\beta$ -hydroxyethyl sulfide (8), and S-methylthioglycolic acid, which lack the onium structure, are likewise inactive in promoting growth. This criterion for methylation activity is further coupled with an enzymic specificity, since many other methylated onium compounds, including arsenocholine (21), N-methylnicotinamide (22), trigonelline (3), and various betaines (3) are ineffective in this respect.

#### SUMMARY

Growth studies on rats fed methionine-choline-free diets supplemented with homocystine together with a number of compounds related to di-

methylthetin show that, in addition to the latter compound, dimethylpropiothetin is a highly active methyl donor. Methylethylthetin is less active and diethylthetin is quite inactive.

Dimethylpropiothetin is an effective lipotropic and kidney antihemorrhagic agent, methylethylthetin is again less active, and diethylthetin shows no protective properties.

S-Methylthioglycolic acid is unable to support growth or to protect animals against renal hemorrhagic damage. Its inability to act as an efficient methyl donor to homocystine has been confirmed by labeling the S-methyl group with deuterium. Inappreciable amounts of the isotope were found to be present in the methyl groups of tissue choline and creatine after 11 days.

The relationship of methyl-donating ability to chemical structure is discussed.

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# BIOSYNTHESIS OF PENICILLINS\*

## VIII. STUDIES WITH NEW BIOSYNTHETIC PENICILLINS ON PENICILLIN RESISTANCE

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It has been reported that the natural penicillins show a similar specificity of action on various microorganisms. Eisman (1) has demonstrated that a *Staphylococcus* which had acquired resistance to benzylpenicillin (penicillin G) was also resistant to *p*-hydroxybenzyl- and 2-pentenylpenicillins (penicillins X and F). However, several investigators have observed that the relative activity of the penicillins was not regular, but varied from organism to organism (*c.g.* (2)). It was of interest to determine whether the activity of the new biosynthetic penicillins (3) would fall in a similar narrow range of variation, or whether such penicillins, containing acyl groups derived from biologically foreign substances, would exhibit a wider range of action.

Resistance of organisms to penicillin has been ascribed to at least two different mechanisms. One of these involves production of penicillinase either as an intracellular or extracellular enzyme. The other does not appear to involve penicillinase. In determining the specificity of action of the new penicillins, tests were, therefore, conducted (*a*) by comparing the rates of reaction of these compounds with penicillinase, and (*b*) by determining their effectiveness against a benzylpenicillin-resistant strain of *Staphylococcus aureus* 209-P which did not produce demonstrable quantities of penicillinase.

### EXPERIMENTAL

*Reactions of Penicillinase with Some Biosynthetic Penicillins*—The manometric method of Henry and Housewright (4) for assaying penicillin proved to be a simple and relatively precise procedure for following the penicillin-penicillinase reactions. The following solutions were used in the determinations.

1. Sodium bicarbonate buffer, pH 7.0, prepared by dissolving 357.1 mg. of sodium bicarbonate (Baker) in 500 ml. of distilled water and equilibrating the solution with a gas mixture of 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>.

\* For paper VII, see Soper, Q. F., Whitehead, C. W., Behrens, O. K., Corse, J., and Jones, R. G., *J. Am. Chem. Soc.*, **70**, 2849 (1948).

2. Penicillinase solution, prepared immediately before use by dissolving 0.7 mg. of penicillinase<sup>1</sup> in 12.5 ml. of the bicarbonate buffer.

3. Penicillin solutions, prepared by dissolving the sodium salt of the penicillin in enough bicarbonate buffer so that 0.5 ml. of solution contained  $1 \times 10^{-5}$  mole of the sodium penicillin. The concentrations of penicillin solutions were checked by bioassay.

Constant volume Warburg respirometers were used. The main compartment of the vessel contained 2.5 ml. of bicarbonate buffer and 0.5 ml.

TABLE I  
*Destruction of Penicillins by Penicillinase*

Penicillin, sodium salt	No of assays	k ratio*
Phenoxyethyl-	2	1 40
$\beta$ -Phenoxyethylmercaptomethyl-	15	1 17
$\beta$ -Bromoallylmercaptomethyl-	4	1 17
$\alpha$ -Thiophenemethyl-	4	1 15
m-Trifluoromethylphenylmercaptomethyl-	2	1 14
Allylmercaptomethyl-	6	1 08
o-Fluorobenzyl-	2	1 07
Ethylmercaptomethyl-	6	1 05
p-Bromobenzyl-	2	1 02
Isopropylmercaptomethyl-	6	1 02
Phenylselenomethyl-	2	1 01
Benzyl-	37	1 00
p-Methoxybenzyl-	1	0 98
Isoamylmercaptomethyl-	9	0 97
n-Butylmercaptomethyl-	9	0 92
n-Propylmercaptomethyl-	6	0 92
m-Fluorobenzyl-	2	0 90
p-Tolylmethyl-	2	0 83
Cyclopentylmethyl-	4	0 79

\* (Rate of reaction of penicillinase and new penicillin)/(rate of reaction of penicillinase with benzylpenicillin). The values represent the average of the several determinations.

of penicillin solution. The system was equilibrated with the gas mixture at 37.5°, closed, and the enzyme solution (0.5 ml.) from the side arm was tipped into the reaction vessel. The rate of carbon dioxide evolution was followed for 100 minutes. Duplicate or triplicate determinations were made on each penicillin in comparison with benzylpenicillin.

The rates of CO<sub>2</sub> evolution varied considerably from one run to another, presumably reflecting differences in enzyme concentration. However, the ratio of the rates of reaction of an experimental penicillin to that of benzyl-

<sup>1</sup> Penicillinase A, furnished by the courtesy of Dr. George E. Ward of the Schenley Laboratories, Inc., Lawrenceburg, Indiana.

penicillin ( $k$  ratio) was reproducible to  $\pm 6$  per cent in all but one case. The  $k$  ratio varied from 0.79 for cyclopentylmethylpenicillin to 1.40 for phenoxymethylpenicillin (cf. Table I). Thus, though the biosynthetic penicillins reacted with penicillinase at significantly different rates, the differences were not great enough to be of therapeutic importance.

*Effect of Some Biosynthetic Penicillins on Benzylpenicillin-Resistant Staphylococcus aureus*—A strain resistant to 200 units per ml. of benzylpenicillin in broth culture was developed by serial transfers of *Staphylococcus aureus* 209-P through broth and on agar plates containing increasing concentrations of penicillin.

TABLE II

*Effectiveness of Biosynthetic Penicillins on Benzylpenicillin-Resistant Strain of Staphylococcus aureus*

Penicillin (Na salt)	Purity	Average relative effectiveness
	per cent	
$\alpha$ -Thiophenemethyl-.....	95	1.7
<i>p</i> -Bromobenzyl-.....	89	1.6
<i>m</i> -Fluorobenzyl-.....	100	1.2
<i>p</i> -Methoxybenzyl-.....	99	1.0
Ethylmercaptomethyl-.....	98	1.0
Benzyl-.....	100	1.0
<i>o</i> -Fluorobenzyl-.....	94	0.96
Cyclopentylmethyl-.....	96	0.62
<i>p</i> -Tolylmethyl-.....	96	0.62
Phenylselenomethyl-.....	98	0.60
<i>n</i> -Propylmercaptomethyl-.....	98	0.54
Isopropylmercaptomethyl-.....	97	0.47
$\beta$ -Bromoallyl-.....	91	0.41
Allylmercaptomethyl-.....	96	0.40
Phenoxymethyl-.....	90	0.35
<i>n</i> -Butylmercaptomethyl-.....	98	0.32
Isoamylmercaptomethyl-.....	94	0.26
$\beta$ -Phenoxyethylmercaptomethyl-.....	98	0.21

Penicillin assays were performed by the plate method (5) with paper disks (6) by use of the resistant strain of *Staphylococcus aureus* as inoculum. An 0.018 M solution of benzylpenicillin (10,000 units per ml.) produced an 18.8 mm. zone.

Concentrations of penicillin were plotted against diameters of the corresponding zones of inhibition. The ratios of moles of benzylpenicillin and biosynthetic penicillins giving equal zones were determined at three or four points on the curve and the average ratios were calculated (cf. Table II).

Generally, the relative activities of the new penicillins decreased as concentrations increased. However, ethylmercaptomethylpenicillin and

*p*-methoxybenzylpenicillin were less effective than benzylpenicillin at lower concentrations but became more effective at 0.045 M (20,000 units per ml.) and 0.026 M (15,000 units per ml.) concentration respectively.

The resistant organism apparently did not produce penicillinase. Filtrates from nutrient broth cultures did not inactivate benzylpenicillin in 4 hours at room temperature. 10 mg. per cent suspensions of cells which had been extracted with acetone and ether did not enable a sensitive strain of *Staphylococcus aureus* to survive in increased concentrations of penicillin.

Table II also gives figures concerning the purity of the penicillins tested. These values represent the ratio of the found analytical value to the calculated analytical value for the analysis which deviated most from the theoretical value. In the penicillins which contained a unique group that could be determined (*i.e.*, OCH<sub>3</sub>, Br, Se), the ratio should be an accurate measure of purity. In other penicillins, for which C, N, or S determinations were utilized, this measure of purity is only approximate, as some possible contaminating materials also may contain these elements.

In spite of the fact that the resistance was induced by subculturing in the presence of benzylpenicillin, this penicillin was one of the most effective in preventing growth of the organism.

No correlations could be made between the action of the new penicillins with penicillinase and the bacteriostatic action of the penicillins on benzylpenicillin-resistant *Staphylococcus aureus*.

The authors express their gratitude to Dr. J. M. McGuire for assistance with media and assays and to E. Brown Robbins for assistance with the manometric work.

#### SUMMARY

1. The rate of destruction of a number of biosynthetic penicillins by penicillinase has been compared with the rate of destruction of benzylpenicillin.

2. The relative effectiveness of a number of biosynthetic penicillins on a strain of benzylpenicillin-resistant *Staphylococcus aureus* has been determined.

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# THE RÔLE OF TRYPTOPHAN IN THE NUTRITION OF DOGS ON NICOTINIC ACID-DEFICIENT DIETS\*

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It has now been definitely established that there exists an interchangeability of nicotinic acid and tryptophan in the nutrition of some animals on diets deficient in the vitamin and containing only suboptimal amounts of the amino acid (1-8).

Because the dog has been employed as the experimental animal in the classical investigations on nicotinic acid deficiency, it seemed of interest to determine whether this same nicotinic acid-tryptophan relationship exists for this species. The results of these nutrition experiments, together with those obtained in the study of the urinary excretion of some nicotinic acid derivatives under these experimental conditions, are presented here.

## EXPERIMENTAL

Twenty-two weanling, mongrel puppies, 7 to 8 weeks old, were used in these studies. The basal ration consisted of casein (Labco) 19, sucrose 66, salts (9) 4, and cottonseed oil 11 parts. All rations were fed *ad libitum* and each dog received in addition, per kilo of body weight per day, 100  $\gamma$  each of thiamine, riboflavin, and pyridoxine, 500  $\gamma$  of calcium pantothenate, and 50 mg. of choline chloride. Folic acid and biotin were administered at a level of 30 and 20  $\gamma$ , respectively, per dog per day. The required amounts of vitamins were given in 20 per cent ethyl alcohol solutions twice weekly. Vitamins A and D were given once weekly in the form of halibut liver oil fortified with viosterol at a level of 3 drops per kilo of body weight. Supplements of casein, zein, and gelatin to the basal diet replaced an equal amount of sucrose.

The 24 hour urine specimens were collected under toluene. The hydrolysis of urine was accomplished by autoclaving at 15 pounds for 15 minutes an aliquot of urine with an equal volume of either 2.0 N  $H_2SO_4$  or NaOH. The nicotinic acid content of the specimens and hydrolysates was determined microbiologically by the method of Snell and Wright (10). N<sup>1</sup>

\* Presented in part at the meetings of the Federation of American Societies for Experimental Biology, Chicago, 1947 (*Federation Proc.*, 6, 422 (1947)). Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Folic acid was generously contributed by the Lederle Laboratories Division, American Cyanamid Company.



Methylnicotinamide was determined by the method of Huff and Perlzweig (11).

### Nutrition Studies

Growth data of dogs on nicotinic acid-deficient diets are presented in Table I. It is evident that upon the institution of the basal diet dogs gain

TABLE I  
*Growth of Dogs on Nicotinic Acid-Deficient Diets*

Diet	Dog No.	Weight gain	Days to reach plateau	Weight gain after nicotinic acid*	
		gm.		gm.	gm. per mg. nicotinic acid
Basal	1 ♂	540	8	960	38
	3 ♀	740	15	1240	38
	9 ♀	520	11	800	26
	10 ♀	600	14	960	33
	11 ♂	520	14	920	27
	16 ♂	460	14	1380	37
	20 ♂	420	8	1000	30
Average . . . .		540	12	1050	33
Basal + 21% zein	6 ♀	620	15	1080	36
	17 ♂	780	18	1200	39
Average . . . . .		700	17	1140	38
Basal + 21% gelatin	5 ♀	560	11	700	22
	18 ♂	630	16	750	25
Average . . . . .		600	14	725	24
Basal + 21% casein	7 ♀	1100	21	1400	46
	19 ♀	1500	24	1650	50
	21 ♂	1450	37		
Average . . . .		1350	27	1525	48

\* A single dose of nicotinic acid at a level of 10.0 mg. per kilo was administered.

an average of 540 gm. before reaching a growth plateau in about 12 days. Thereafter drastic weight loss is usually rapid and other symptoms of blacktongue, characterized by inflammation of the gums, palatine redness, and diarrhea, usually develop if therapy is withheld. To reduce the loss of dogs, nicotinic acid or tryptophan was usually administered after the dogs refused food for 48 hours. Frequently, severe symptoms had al-

ready developed. When one therapeutic dose of nicotinic acid was given at a level of 10.0 mg. per kilo of body weight, the dogs resumed intake of food within 24 hours and gained on an average 1140 gm. before reaching another weight plateau. This amounts to 33 gm. gain in weight per mg. of nicotinic acid given.

The development of nicotinic acid deficiency in dogs on the basal diet is not prevented or significantly altered by the inclusion of the tryptophan-deficient proteins, zein or gelatin, in the diet. Two dogs receiving the basal ration supplemented with 21 per cent zein gained an average of 700 gm. before reaching a growth plateau in 17 days. With a therapeutic dose of nicotinic acid 1140 gm. were added to the body weight, or an average of 38 gm. per mg. of nicotinic acid administered. The two dogs on the basal diet supplemented with 21 per cent gelatin show similar increases in body weight before growth plateau. With nicotinic acid, however, the growth response is somewhat smaller, as is evident from the smaller value of gm. gained per mg. of nicotinic acid given.

The addition of 21 per cent casein to the basal diet does not prevent the onset of nicotinic acid deficiency, but significantly delayed its appearance as judged by the much larger weight gain before the weight plateau was reached and the longer period of time necessary to reach the plateau. In two of these animals this plateau persisted for 17 and 21 days before drastic decline in weight occurred. The plateau of animals on the basal diet lasted from 3 to 12 days. The response to nicotinic acid is also larger than that observed on the other diets. In the third animal, after a gain of 1450 gm., the growth plateau persisted for 30 days. Nicotinic acid was given when there was no indication of drastic weight loss. At first the animal gained weight steadily and during the next 60 days growth was erratic, but there was no significant tendency for the animal to reach a weight plateau.

With supplementary L-tryptophan at a level of 0.5 per cent, one dog (Fig. 1, Dog 2) received complete protection against the onset of nicotinic acid deficiency for an experimental period of 60 days. This animal grew at a rate comparable to that of animals receiving nicotinic acid at a level of 500  $\gamma$  per kilo of body weight per day in addition to the basal diet. Inasmuch as a casein supplement of 21 per cent to the basal diet did not afford protection, the supplement was increased to 42 per cent, at which level the tryptophan content of the entire diet was equivalent to that of the basal diet supplemented with 0.5 per cent L-tryptophan.<sup>1</sup> This high level of protein in the diet afforded complete protection to two dogs for periods of 74 and 100 days, the length of the experimental periods (Fig. 1, Dog 12). In these periods the dogs gained 8.1 and 9.0 kilos in body weight.

<sup>1</sup> The tryptophan content of casein was taken as 1.2 per cent (12).

Inasmuch as further experiments with L-tryptophan were inadvisable because of the cost of the natural amino acid, DL-tryptophan was employed in the remaining experiments of the study. As with the L form, the racemic amino acid at a level of 0.5 per cent prevents the occurrence of nicotinic acid deficiency in the dog (Fig. 1, Dog 8). Levels of 0.3, 0.2, and 0.1 per

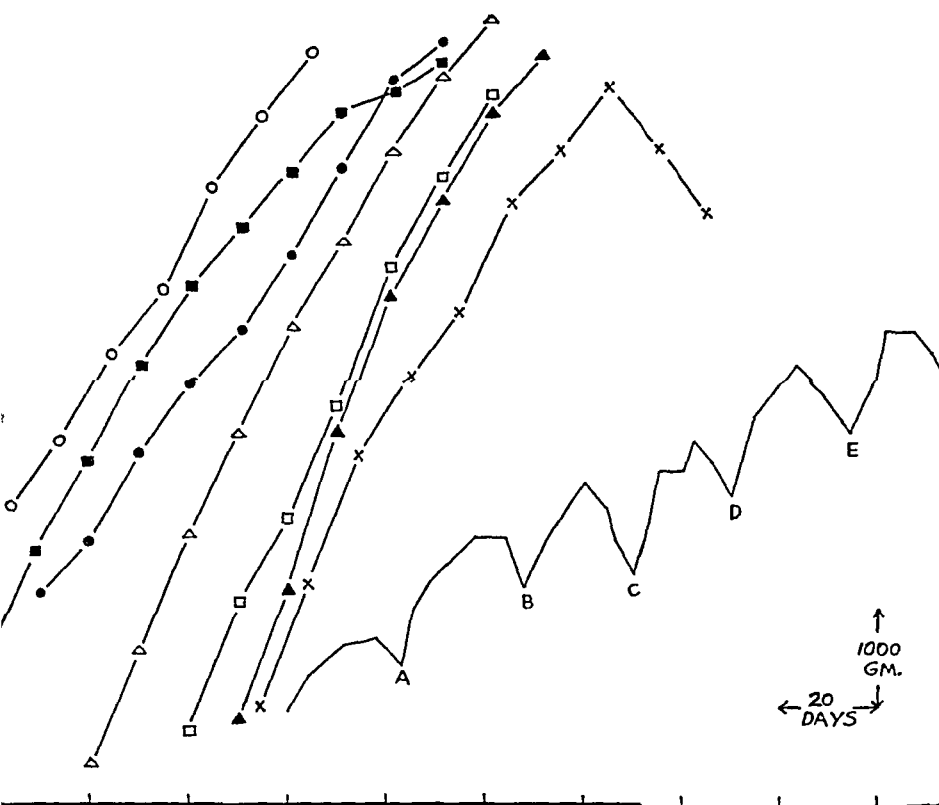


FIG. 1. The growth of dogs on various diets. O, Dog 2 ♀, basal diet + 0.5 per cent L-tryptophan; ●, Dog 8 ♂, basal + 0.5 per cent DL-tryptophan; ▲, Dog 15 ♀, basal + 0.3 per cent DL-tryptophan; □, Dog 14 ♀, basal + 0.2 per cent DL-tryptophan; X, Dog 13 ♂, basal + 0.1 per cent DL-tryptophan; △, Dog 12 ♀, basal + 42 per cent casein; ■ Dog 4 ♂, basal + 500  $\gamma$  of nicotinic acid per kilo of body weight daily. The lower right-hand curve represents Dog 3 ♀, basal diet; 33 mg. of nicotinic acid given at A, C, and E; 3.96 and 5.66 gm. of L-tryptophan at B and D, respectively.

cent were then fed to determine the minimal protective level. It is apparent that this minimum must be less than 0.1 per cent, since dogs at this level are fully protected (Fig. 1, Dog 13). After a gain in body weight of 6.3 kilos distemper appeared in one dog and the experiment was terminated. It is, however, apparent that 0.1 per cent DL-tryptophan affords protective

action. It is of interest to note that, although the basal diet supplemented with 21 per cent casein containing a total of 0.48 per cent tryptophan<sup>1</sup> is ineffective in preventing nicotinic acid deficiency, the basal diet supplemented with 0.1 per cent DL-tryptophan containing a total of 0.28 per cent tryptophan<sup>2</sup> is effective in this respect. One must assume, therefore, under these experimental conditions that supplementary tryptophan must be metabolized in a manner different from tryptophan present as an integral part of the protein molecule. It also appears unlikely that tryptophan present in the protein molecule with other amino acids is more readily utilized for the synthesis of tissue protein, a process which would remove it as a source of nicotinic acid synthesis. The protein requirement of the dog is met by a level of 19 per cent casein, because supplementary nicotinic acid promotes normal growth under these conditions (Fig. 1, Dog 4). This leaves unaccounted for the metabolic route of the protein tryptophan in the 21 per cent casein supplement. One might consider that the free amino acid is more rapidly absorbed than protein tryptophan because of the delay necessitated by the hydrolytic process in the gastrointestinal tract and thereby metabolized in the absence of large amounts of other amino acids present in the protein. In this respect it would be of interest to compare the protective action of supplementary casein with that of an enzyme hydrolysate of casein.

At the present time there is not sufficient evidence to account for the protective action of high levels of supplementary casein (42 per cent). It is certainly not related to the nicotinic acid content of the protein *per se*. By microbiological assay a casein hydrolysate prepared with trypsin contains only 0.17  $\gamma$  of nicotinic acid per gm. of casein. The two dogs, each consuming an average of 300 gm. of diet daily, would obtain only 31  $\gamma$  of nicotinic acid in this manner. With an initial body weight of 3.0 kilos, the intake would be only 10  $\gamma$  per kilo of body weight. This is less than one-twentieth of the minimal requirement (13).

In view of the fact that L- and DL-tryptophan had protective action at the levels fed in these experiments, it was of interest to determine the curative potency of the D and L forms in terms of nicotinic acid. This was performed by comparing the growth-promoting effect of a single dose of the L- and DL-amino acid in nicotinic acid-deficient dogs, which had been standardized with known amounts of nicotinic acid (13). The procedure was altered in that the assay of the amino acid was preceded and followed by a standardization response to nicotinic acid. An example of this assay is shown in Fig. 1. The results of these experiments are presented in Table

<sup>2</sup> The calculation includes the tryptophan content of the casein of the basal diet and in addition one-half of the supplementary DL-tryptophan, as only the L form is utilized for nicotinic acid synthesis (see Table II).

II. It is apparent that only the natural form is utilized for nicotinic acid synthesis. The curative effect of L-tryptophan failed in three assays. In one the administration of nicotinic acid promptly stopped the drastic weight loss and a growth response was then obtained which was in excess of that expected from nicotinic acid alone. In the other two animals amounts of nicotinic acid as high as 500 mg. failed to check the rapid decline. In one of these nasal hemorrhage was observed. The administration of 500 mg. of ascorbic acid and 2.0 mg. of 2-methyl-1,4-naphthoquinone intramuscularly was without effect and the two animals died in an extremely emaciated condition 9 days later.

TABLE II

*Nicotinic Acid Equivalence of L- and DL-Tryptophan\**

Values in mg. per gm. of amino acid.

Dog. No.	L-Tryptophan	DL-Tryptophan
3	5.6 6.1	Plateau† 3.0
5	6.1	Plateau
7	9.0	"
9	10.0 8.1	" 5.1
11	7.7	
16	8.2	3.7

\* Tryptophan was administered at a level of 1200 mg. per kilo of body weight in two portions during a 24 to 48 hour period. To prevent vomiting of the supplement, the amino acid was ground with an equal weight of sucrose and given either in gelatin capsules or as a paste.

† The term plateau indicates that the drastic weight loss ceased and that the body weight was maintained for at least 7 days.

### *Nicotinic Acid Excretion Studies*

In the rat the administration of tryptophan is followed by the urinary excretion of relatively large amounts of nicotinic acid derivatives (14-18). In Table III are given the results obtained in the dog. Change of diet from commercial dog food to the synthetic diet is reflected in a rapid fall in the excretion of the nicotinic acid derivatives measured. This is less marked in the nicotinic acid values obtained after acid hydrolysis. In fact, by the 2nd day complete recovery has been obtained. Thereafter the values rise steadily to rather large levels. By contrast nicotinic acid values obtained in untreated or alkaline-hydrolyzed urine after the initial drop regain only a third of their original values. Since nicotinic acid and its amide have equal activities for the assay organism, *Lactobacillus arabi-*

*nosus*, and *nicotinuric acid* does not require preliminary hydrolysis to show the same activity as its theoretical equivalent of nicotinic acid (10), it appears that after the administration of tryptophan to dogs, as in rats (15), there is excreted a nicotinic acid precursor which is converted to nicotinic acid by acid hydrolysis. This substance makes up the major portion of the total nicotinic acid derivatives excreted. By comparison *N*<sup>1</sup>-methylnicotinamide, which is the major excretory substance in animals on a commercial animal ration, is only temporarily excreted in large amounts in dogs on synthetic diets containing tryptophan (days 3 to 8). Following this period the values are not in excess of those found in dogs receiving nico-

TABLE III  
*Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet Supplemented with 0.5 Per Cent L-Tryptophan*

Days on diet	Nicotinic acid			<i>N</i> <sup>1</sup> -Methyl-nicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0*	655	720	692	2650
1	100	414	137	1072
2	84	700	64	206
3	208	1240	208	1460
5	205	1120	200	1410
8	302	1560	305	2710
16	153	1848	113	861
25	120	1830	103	600
31	225	2340	182	709
40	191	2170	191	615
46	192	2240	222	579
56	240	2660	178	573

\* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

tinic acid (Table IV). In the rat tryptophan stimulates the excretion of relatively large amounts of nicotinic acid, nicotinic acid precursor, and *N*<sup>1</sup>-methylnicotinamide, whereas in the dog only the excretion of the acid-hydrolyzable nicotinic acid precursor is marked. The pattern of excretion of nicotinic acid derivatives is markedly altered in dogs when the commercial ration is replaced by the basal diet supplemented with nicotinic acid (Table IV). In the former the excretion of nicotinic acid is the same regardless of the treatment of the urine, indicating that the substances excreted resemble the simple derivatives, nicotinic acid, *nicotinamide*, or *nicotinuric acid*. *N*<sup>1</sup>-Methylnicotinamide accounts for 87 per cent of the total nicotinic acid derivatives excreted. By comparison, on the synthetic

diet the values of nicotinic acid excretion are dependent on the treatment of urine before assay. Similar values are obtained in untreated or alkaline-hydrolyzed urine. With acid treatment the apparent nicotinic acid is increased by as much as a 3-fold factor. This difference may indicate that the dog on the synthetic ration excretes small amounts of the acid-hydrolyzable precursor encountered in animals receiving tryptophan.<sup>3</sup> The excretion of N<sup>1</sup>-methylnicotinamide is also markedly reduced in the change of ration. The 6-pyridone of N<sup>1</sup>-methylnicotinamide (19, 20) may also play an important rôle in excretion of nicotinic acid derivatives in the dog. It has recently been reported that this substance is the major pathway of

TABLE IV

*Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet and 0.5 Mg. of Nicotinic Acid per Kilo of Body Weight per Day*

Days on diet	Nicotinic acid			N <sup>1</sup> -Methyl-nicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0*	588	573	513	3880
1	394	470	338	1530
2	88	224	75	234
3	100	220	80	233
5	114	275	88	350
8	114	326	93	341
16	142	324	121	412
25	118	330	81	442
31	201	375	213	505
40	155	344	121	569
50	193	400	152	502
61	210	470	185	613

\* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

excretion of dietary nicotinic acid in the human (21). Similarly, it may account for the major fraction of methylated derivatives of nicotinic acid in dogs receiving tryptophan or nicotinic acid.

The excretion of nicotinic acid derivatives in a dog on a high casein diet is shown in Table V. The protective action of this diet is apparently related to the synthesis of nicotinic acid as judged by the increased excre-

<sup>3</sup> The nature of the nicotinic acid fraction in the rat is similarly affected by the replacement of commercial animal ration with a synthetic diet. On the former, rats excrete nicotinic acid, the value of which is the same regardless whether the urine has been hydrolyzed with acid or alkali or left untreated before assay. Upon change to the synthetic diet, the urinary nicotinic acid is always somewhat greater in acid-hydrolyzed specimens.

TABLE V

*Urinary Excretion of Nicotinic Acid Derivatives in Dog Receiving Basal Diet Supplemented with 42 Per Cent Casein*

Days on diet	Nicotinic acid			N <sup>1</sup> -Methyl-nicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0*	598	630	610	2700
1	102	585	107	1100
2	161	962	177	649
3	181	846	180	1422
5	210	1039	207	1666
8	167	880	189	1166
25	255	763	261	1607
27	244	807	240	2431

\* Excretion values on this day represent those of the animal on a commercial animal ration. Thereafter, the experimental diet was instituted.

TABLE VI

*Effect of L-Tryptophan and Nicotinic Acid on Urinary Excretion of Nicotinic Acid Derivatives in Dog with Blacktongue (Dog 9)*

Days on diet	Nicotinic acid			N <sup>1</sup> -Methyl-nicotinamide
	No hydrolysis	Acid hydrolysis	Alkaline hydrolysis	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0*	140	366	108	52
1†	84	472	66	186
2†	172	890	156	1150
3	134	950	107	1080
4	136	452	112	469
5	110	356	81	482
6	95	305	70	96
7	107	250	90	104
8	112	278	88	91
0*	108	228	91	107
1†	4210	4220	4400	3630
2	119	209	112	656
3	140	270	127	566
4	98	161	84	140
5	153	290	131	391

\* Excretion values on these days represent those of the animal in a nicotinic acid-deficient state.

† 2.0 gm. of L-tryptophan were given in a capsule.

‡ 35 mg. of nicotinic acid were given in a capsule.



tion of the acid-labile precursor and N<sup>1</sup>-methylnicotinamide. The appearance of the latter in amounts greater than that found either in dogs on the basal diet supplemented with nicotinic acid (Table IV) or in the over-all excretion period in dogs receiving tryptophan will require further study for explanation.

In the nicotinic acid-deficient dog relatively large amounts of tryptophan can stimulate the increased excretion of the acid-hydrolyzable precursor of nicotinic acid and N<sup>1</sup>-methylnicotinamide (Table VI). In six studies four animals have shown these increased values. The failure of the other two has been interpreted as retention of the nicotinic acid synthesized from tryptophan. It would be well also to consider the efficiency of this synthesis in the deficient animal. When receiving therapeutic amounts of nicotinic acid, the deficient dog excretes only 23 per cent of the administered dose as nicotinic acid and N<sup>1</sup>-methylnicotinamide. The nicotinic acid precursor does not appear to be excreted in significant amounts after the administration of nicotinic acid as judged by the similar values of nicotinic acid excretion obtained in untreated and acid- and alkaline-hydrolyzed urine. This may imply that the precursor represents an intermediate in the synthesis of nicotinic acid and not a mechanism to remove a surplus of the vitamin in the body. Its excretion in dogs receiving tryptophan would indicate that its conversion to nicotinic acid is at a lower rate than the methylation of the nicotinic acid to N<sup>1</sup>-methylnicotinamide or more likely the 6-pyridone derivative.

#### DISCUSSION

The interchangeable rôle of nicotinic acid and tryptophan has already been demonstrated in the nutrition of the rat (1, 2, 5-7), mouse (4), chick (3), and pig (8, 22). Our results indicate that this relationship also exists for the dog. Urinary excretion studies lend support to the rôle of tryptophan as the biological precursor of nicotinic acid (7, 14-18).

The level of casein in the diet necessary to abolish the requirement for nicotinic acid varies from 20 to 25 per cent for the rat (7, 23) and the pig (8, 22) to a value in excess of 40 per cent for the dog as reported here. If these latter results are applicable to human nutrition, some question is obviously raised as to the relative importance of protein and dietary nicotinic acid in meeting the nicotinic acid requirement. It would appear rather unlikely that this level of protein is within the average dietary experience (24). This would emphasize the importance of nicotinic acid in diets of moderate protein content in which the protein is derived to a large extent from animal sources (24). These foods contain large amounts of the vitamin (25).

Rather large quantities of milk have been successful in the treatment of

pellagra (26). In the rat its beneficial action has been ascribed to its protein content, since the amount of nicotinic acid present is very low (2). In our own studies 5 ml. of milk per day were ineffective in preventing growth retardation in rats on low protein diets supplemented with gelatin.<sup>4</sup> Experiments with the nicotinic acid-deficient dog have indicated that milk has a greater beneficial action than can be ascribed to its nicotinic acid content as determined microbiologically (13). In our own hands this procedure has given rather unreliable results.

The presence of a substance in milk similar to nicotinic acid in its biological activity should be considered in view of the fact that milk, the major food consumed after birth, contains little nicotinic acid. On a dry basis human milk contains only 10 per cent protein (27), which is insufficient for nicotinic acid synthesis in the rat and pig, and certainly a quantity considerably less than that required by the dog. Salmon (28) has shown that the nicotinic acid requirement of the rat is abolished on low protein diets containing 30 per cent fat. In view of the fact that on the dry basis milk contains 28 per cent fat, we may have here a possible explanation for the absence of nicotinic acid deficiency in the nursing young.

#### SUMMARY

The interchangeable rôle of nicotinic acid and tryptophan in the nutrition of dogs has been studied. At a level of 21 per cent in a nicotinic acid-deficient ration, gelatin, zein, or casein does not prevent the onset of nicotinic acid deficiency in the dog. However, when the casein supplement was increased to 42 per cent, complete protection was obtained.

Supplementary L- or DL-tryptophan at the 0.5 per cent level similarly prevents the occurrence of the deficiency syndrome. The minimal protective level of DL-tryptophan is less than 0.1 per cent, as dogs on the deficient ration supplemented with 0.3, 0.2, or 0.1 per cent of the amino acid grow at the normal rate and are without deficiency symptoms during the experimental period.

The nicotinic acid equivalence of L- and DL-tryptophan has been determined in nicotinic acid-deficient animals, which have been standardized with known amounts of nicotinic acid. By this method it has been found that only the natural isomer is utilized for nicotinic acid synthesis.

The urinary excretion of some nicotinic acid derivatives in the dog has been studied. The administration of L-tryptophan results in the excretion of an acid-hydrolyzable precursor of nicotinic acid, which has previously been reported present in the urine of rats under similar conditions. There is only a temporary increase in urinary N<sup>1</sup>-methylnicotinamide. Dogs on rations supplemented with 42 per cent casein also excrete larg un.

<sup>4</sup> Unpublished results.

of the precursor and N<sup>1</sup>-methylnicotinamide than did dogs growing normally on the basal ration supplemented with nicotinic acid. The results of the excretion and nutrition studies are interpreted as indicating that the synthesis of nicotinic acid from tryptophan does take place in this species.

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# FURTHER STUDIES ON THE EFFECT OF SOME AMINO ACIDS ON THE GROWTH AND NICOTINIC ACID STORAGE OF RATS ON LOW CASEIN DIETS\*

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(Received for publication, July 23, 1948)

In a previous communication from this laboratory it was shown that the inclusion of an amino acid mixture containing histidine, valine, threonine, and lysine in a low protein diet produced marked depression of growth in rats (1). The addition of either nicotinic acid or tryptophan not only corrected the growth retardation, but permitted normal growth, which was not possible in the absence of these amino acids. That lysine was not the amino acid responsible for the growth depression was evident from the fact that its omission from the amino acid mixture did not significantly alter the results obtained.

In the present paper the investigation has been continued to determine which amino acids are growth depressants and which are required by the rat for normal growth on low casein diets supplemented with nicotinic acid or tryptophan. Data are presented which indicate that threonine is the amino acid effective in both categories.

## EXPERIMENTAL

Wistar strain rats, 22 days old, were used in these experiments. The basal diet consisted of casein (Labco) 9, sucrose 82, salts (2) 4, L-cystine 0.2, cottonseed oil 3, and cod liver oil 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 1.0 mg., riboflavin 1.0 mg., pyridoxine 1.0 mg., calcium pantothenate 2.0 mg., choline chloride 200 mg., 2-methyl-1,4-naphthoquinone 0.5 mg., inositol 10 mg., biotin 0.02 mg., and folic acid 0.2 mg.  $\alpha$ -Tocopherol was administered at a level of 1.0 mg. per rat per week. Supplements of amino acids replaced an equal amount of sucrose in the diet. At first they were incorporated in the diet as pairs and later their effects were studied individually.

The nicotinic acid content of muscle and liver was determined microbiologically (3) on tissue extracts prepared by autolysis aided by taka-diastase and papain (4).

\* Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Folic acid and biotin were generously contributed by the Lederle Laboratories Division, American Cyanamid Company, and Merck and Company, Inc., respectively.

From the results in Table I it is evident that growth on the basal ration can be improved to a limited, yet reproducible, extent by the addition of either nicotinic acid or tryptophan. This is reflected in an increased nicotinic acid content of muscle and liver, although the amino acid is much more effective in this respect. Under these experimental conditions it is possible to maintain nicotinic acid storage that is near normal with supplementary tryptophan in spite of suboptimal growth (Diets 3 to 5).

When threonine and valine are added to the basal ration, there is observed a marked growth depression, which is not reflected in a concomitant decrease in nicotinic acid storage (Diet 6). On the contrary, values are obtained which are usually in excess of those found for rats on the basal diet alone. This may be explained on the basis of a decreased demand on the nicotinic acid stores of the rats on threonine-containing diets, because of the small amount of new tissue laid down. Supplementary nicotinic acid or tryptophan not only corrects the inhibition, but permits normal growth, which is not possible in the absence of these amino acids (Diets 7 and 8). Liver nicotinic acid is nearly normal in animals receiving dietary nicotinic acid, whereas there is no considerable change in muscle storage. The inability of dietary nicotinic acid to raise substantially muscle storage toward normal has been observed in all threonine-containing diets (Nos. 7, 13, and 16), in spite of reasonably good growth. With supplementary tryptophan muscle nicotinic acid is in the normal range. Liver storage is considerably above normal, a finding previously reported (1). Perhaps this indicates an accumulation in the liver of either nicotinic acid or intermediates in the synthesis of nicotinic acid from tryptophan, which can replace the nicotinic acid requirement of the test organism, *Lactobacillus arabinosus*, employed in the microbiological assays. Apparently not involved is the acid-hydrolyzable derivative of nicotinic acid previously reported present in the urine of rats receiving tryptophan (1, 5).

The amino acid pair containing valine and histidine, when added to the basal ration, does not significantly alter either the growth or nicotinic acid storage of rats on the basal ration alone (Diet 9). Supplementary nicotinic acid or tryptophan produces the same effects with this diet as with the basal diet alone (Diets 10 and 11).

With threonine and histidine there is obtained a growth inhibition which resembles that observed in animals on the diet containing threonine and valine (Diet 12). A supplement of nicotinic acid or tryptophan also produces similar effects in regard to growth and nicotinic acid storage (Diets 13 and 14). In consideration of the results obtained with diets containing pairs of amino acids, it is apparent that growth inhibition is observed only when threonine is one member of the pair. In addition the basal ration contains only suboptimal amounts of this amino acid, because when it is

added to the ration, either nicotinic acid or tryptophan can stimulate optimal growth.

TABLE I  
*Growth and Muscle and Liver Nicotinic Acid of Rats on Various Diets*

Diet No.	No. of rats	Diet*	Gain per wk. for 4 wks.	Nicotinic acid	
				Muscle	Liver
			gm.	$\gamma$ per gm.	$\gamma$ per gm.
1	7	Stock	25 (22-29)†	74 (61-85)†	159 (132-185)†
2	7	20% casein	25 (21-33)	78 (70-85)	186 (167-206)
3	11	Basal	9 (8-12)	38 (28-52)	86 (57-114)
4	11	" + 2.0 mg. % nicotinic acid	13 (10-16)	73 (64-90)	111 (63-165)
5	11	" + 0.2 % L-tryptophan	14 (9-17)	79 (65-94)	150 (70-227)
6	8	" + threonine + valine	2 (0-3)	46 (34-64)	137 (64-217)
7	8	Diet 6 + 2.0 mg. % nicotinic acid	22 (18-27)	50 (44-59)	152 (150-166)
8	8	" 6 + 0.2 % L-tryptophan	24 (21-28)	77 (61-87)	228 (171-303)
9	8	Basal + valine + histidine	10 (8-12)	37 (30-48)	90 (43-110)
10	8	Diet 9 + 2.0 mg. % nicotinic acid	14 (11-15)	68 (56-80)	109 (67-138)
11	8	" 9 + 0.2 % L-tryptophan	13 (10-17)	80 (63-92)	144 (77-208)
12	8	Basal + threonine + histidine	2 (1-3)	45 (28-74)	125 (77-164)
13	8	Diet 12 + 2.0 mg. % nicotinic acid	19 (15-25)	53 (47-61)	146 (111-164)
14	8	Diet 12 + 0.2 % L-tryptophan	23 (20-25)	86 (77-95)	222 (190-254)
15	9	Basal + threonine	3 (0-6)	41 (23-67)	131 (93-200)
16	9	Diet 15 + 2.0 mg. % nicotinic acid	19 (16-24)	45 (30-58)	141 (106-186)
17	9	Diet 15 + 0.2 % L-tryptophan	22 (19-27)	70 (56-85)	229 (179-281)
18	8	Basal + valine	9 (8-12)	42 (26-63)	82 (61-109)
19	8	Diet 18 + 2.0 mg. % nicotinic acid	13 (10-15)	64 (44-81)	99 (59-122)
20	8	Diet 18 + 0.2 % L-tryptophan	9 (9-11)	78 (60-88)	127 (83-195)
21	8	Basal + histidine	9 (8-10)	40 (31-60)	84 (58-131)
22	8	Diet 21 + 2.0 mg. % nicotinic acid	12 (9-15)	62 (54-70)	96 (88-107)
23	8	Diet 21 + 0.2 % L-tryptophan	12 (8-16)	79 (65-92)	141 (88-178)
24	5	Basal + phenylalanine	9 (7-11)	41 (34-45)	84 (73-90)
25	5	Diet 24 + 2.0 mg. % nicotinic acid	12 (8-14)	63 (50-71)	101 (71-123)
26	5	Diet 24 + 0.2 % L-tryptophan	12 (8-14)	80 (64-90)	138 (116-184)

\* The stock ration consists of commercial animal food supplemented liberally with milk, lettuce, and carrots. The levels of supplementary amino acids are DL-threonine 0.4 per cent, DL-valine 0.3 per cent, L-histidine monohydrochloride 0.25 per cent, and DL-phenylalanine 0.25 per cent.

† The values in parentheses represent the range.

The effects of the individual amino acids were then studied to determine whether the presence of either valine or histidine was required for the

activity of threonine. It is evident that the growth-depressing action of the amino acid mixture is entirely confined to threonine. Valine and histidine are without effect individually, as they are when included together in the basal ration (Diets 18 to 23). It is difficult to explain the inability of tryptophan to produce in the presence of valine the limited growth response observable on the basal ration plus tryptophan (compare Diets 20 and 5). With supplementary nicotinic acid or tryptophan, threonine alone is required for reasonably good growth (Diets 16 and 17). It is of interest to note that at the level fed nicotinic acid is a little less effective than tryptophan. When the requirement of tryptophan is divided between that necessary for protein synthesis and that needed for nicotinic acid synthesis, it may well be that the amount of the amino acid present in the basal ration alone is not quite sufficient for the former process when nicotinic acid and threonine are included in the ration. When nicotinic acid is replaced by tryptophan, this requirement is easily met. At smaller growth rates, in which protein synthesis is already limited by the amount of threonine present in the basal ration, these differences disappear.

#### DISCUSSION

Krehl *et al.* (5, 6) first observed growth retardation in rats on low protein diets containing large amounts of corn. The addition of either tryptophan or nicotinic acid corrected the deficiency and permitted normal growth to proceed. Briggs extended this observation to the chick and further demonstrated that a similar depression could be obtained by the addition of gelatin to a purified diet (7, 8). In this work the inhibitory effect was not noted when gelatin was replaced by arginine and glycine. Later work from this laboratory indicated that the two amino acids were growth-inhibitory under somewhat altered conditions (9). The addition of alanine furthered the depressant effect. The feeding of an amino acid mixture, simulating the relative occurrences of some amino acids in gelatin, produced an inhibition observed with the protein itself. The omission of arginine and glycine from the mixture permitted good growth. In more recent results Anderson and Briggs observed that the inhibitory effect of some seventeen amino acids varied from the rather deleterious effect of methionine to a 20 per cent inhibition by valine (10).

In extension of their original observations the University of Wisconsin workers have found that it was not necessary to postulate a specific pellagra-genic agent in corn for the rat, at least, because the deficiency syndrome could be duplicated with non-corn rations by the addition of tryptophan-deficient proteins, such as zein or gelatin, or acid-hydrolyzed proteins to a nicotinic acid-deficient diet low in tryptophan (11). Of a number of amino acids tested for growth inhibition, glycine was particularly effective (12). Supplementary nicotinic acid removed the inhibition, but normal growth

was not obtained. The deleterious effect of acid-hydrolyzed casein could be reproduced with a mixture of amino acids made to simulate the hydrolysate. That this inhibition was not due to glycine and alanine *per se* was evident from the fact that their omission from the mixture did not prevent growth inhibition (13).

Beginning with the suggestion that the usual 9 per cent casein diet is deficient in a number of amino acids (14), the present authors observed that the addition of a mixture of amino acids containing lysine, valine, threonine, and histidine to a low protein diet produced marked growth retardation in rats (1). Supplementary nicotinic acid or tryptophan not only corrected the deficiency, but also produced good growth, which was not possible in the absence of these amino acids. The omission of lysine from the mixture did not significantly alter the data. The results of the present report indicate that of the remaining members of the mixture valine and histidine do not alter growth significantly. Threonine alone is responsible for the growth inhibition by the mixture. Furthermore, it is revealed that this amino acid is the limiting factor for growth on the low casein diet. Recently, this latter observation has also been made by Griffith (15).

Salmon (16) in his studies has stated that the primary deficiency on low casein diets was that of labile methyl groups and, secondly, nicotinic acid. Only after the requirement of the vitamin was satisfied, could a deficiency of sulfur amino acid be demonstrated. Under our conditions, in which the basal low protein diet is already supplemented with choline and cystine, the growth-promoting action of nicotinic acid is limited only by the sub-optimal amounts of threonine in the basal ration.

Niven *et al.* (17) have observed marked growth retardation in rats receiving phenylalanine and tyrosine. The addition of relatively large amounts of nicotinic acid or tryptophan corrected only in part the deficiency syndrome. Our results indicate that at a 0.25 per cent level phenylalanine does not have inhibitory action, nor can it promote good growth with supplementary nicotinic acid or tryptophan (Diets 24 and 25).

The nature of the growth-depressing action of threonine is obscure. Whether it represents a specific antagonism between nicotinic acid and the amino acid, or the result of the resolution of a multiple deficiency into one involving a single essential substance, is at present unknown. In regard to the former, one may consider the curious antagonism between threonine and muscle nicotinic acid as reported here.

#### SUMMARY

It was originally observed that an amino acid mixture containing lysine, valine, threonine, and histidine produced in rats on a low casein ration growth retardation which was prevented by either



tinic acid or tryptophan. The omission of lysine from the mixture did not significantly alter the results. Of the remaining components of the mixture, histidine and valine are without effect on growth. Threonine alone produces the growth retardation originally observed with the amino acid mixture. Supplementary nicotinic acid or tryptophan not only corrects the inhibition, but permits good growth, which is not possible in the absence of threonine.

The inhibition by threonine is not accompanied by a decrease in the storage of nicotinic acid in the liver or muscle. The values are usually in excess of those found on the basal diet alone. With nicotinic acid or tryptophan the vitamin storage in the liver is increased to a nearly normal level for the former and to a value considerably in excess of the normal for the latter supplement.

The authors are grateful to Mr. Horace Hayes for the care of animals employed in this investigation.

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# THE NICOTINIC ACID CONTENT OF TISSUES OF RATS ON CORN RATIONS\*

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(Received for publication, July 23, 1948)

With the demonstration that nicotinic acid was a dietary essential for the dog (1), pig (2), monkey (3), chick (4), and man (5-9), it was rapidly shown that the nicotinic acid or pyridine nucleotide content of some tissues is markedly decreased in the deficient animal. Of the tissues studied only voluntary muscle and liver have shown subnormal values in the dog (10-14), pig (14), and chick (15). In pellagra the coenzyme content of muscle decreases as the deficiency progresses (16). Normal values are obtained for erythrocytes (13, 16, 17).

Prior to 1945 no specific disease due to nicotinic acid deficiency was demonstrable in the rat. There was evidence, however, that the rat could synthesize a sufficient amount of the vitamin for growth (18-20). In 1945 Krehl *et al.* (21, 22) observed in rats on low casein diets containing corn a growth retardation, which was prevented by either nicotinic acid or tryptophan. It seemed desirable, therefore, to compare the effects of nicotinic acid deficiency in the rat on the tissue content of this vitamin with those reported for other species. The results of this investigation are reported here.

## EXPERIMENTAL

Wistar strain rats, 21 to 24 days old, were used in these experiments. The synthetic diet consisted of casein (Labco) 15, sucrose 76, cottonseed oil 3, salts (23) 4, cystine 0.5, and cod liver oil 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 2.0 mg., riboflavin 2.0 mg., pyridoxine 2.0 mg., calcium pantothenate 4.0 mg., and choline chloride 1.0 gm. The basal ration was prepared by mixing 60 parts of the above diet with 40 parts of corn grits.

Tissue specimens were prepared for analysis by autoclaving the finely ground material with 1.0 N  $H_2SO_4$  for 30 minutes at 15 pounds. The hot extracts were neutralized to pH 4.5 with brom-cresol green as indicator,

\* Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Crystalline vitamins and DL-tryptophan were generously contributed by Merek and Company, Inc., and Winthrop-Stearns, Inc., respectively.

cooled to room temperature, filtered, and neutralized to pH 6.8 with bromthymol blue as indicator. 1.0 ml. of blood obtained by cardiac puncture was laked with 50 ml. of distilled water and autoclaved with 5.0 ml. of 2.0 N  $\text{H}_2\text{SO}_4$ . From this point the preparation followed the procedure employed for the solid tissues. The nicotinic acid content of the extracts was determined microbiologically by the method of Snell and Wright (24).

TABLE I

*Nicotinic Acid Content of Tissues of Rats on Corn Rations*

Nicotinic acid content expressed as micrograms per gm. of wet tissue or micrograms per ml. of blood. Values in parentheses represent the range.

Diet 1, stock ration; Diet 2, basal; Diet 3, basal + 100  $\gamma$  of nicotinic acid per rat daily; Diet 4, basal + 0.5 per cent L-tryptophan; Diet 5, basal + 200  $\gamma$  of N-methylnicotinamide per rat daily.

	Diet 1, 7 rats	Diet 2, 12 rats	Diet 3, 13 rats	Diet 4, 11 rats	Diet 5, 12 rats
Weight change, gm.	+190 (155-220)	+26 (2-77)	+180 (133-218)	+192 (154-213)	+39 (8-72)
Brain	47 (41-50)	30 (20-35)	48 (40-54)	50 (37-55)	
Heart	114 (108-119)	105 (83-122)	107 (70-142)	105 (90-119)	107 (80-115)
Lung	48 (42-52)	43 (37-45)	46 (42-52)	50 (43-55)	
Spleen	58 (55-67)	57 (43-64)	61 (55-70)	63 (55-73)	
Muscle	78 (73-84)	45 (16-60)	77 (55-97)	81 (70-92)	40 (18-74)
Kidney	87 (80-92)	84 (66-104)	101 (81-130)	101 (80-119)	84 (61-94)
Liver	157 (130-173)	105 (57-136)	160 (128-202)	214 (173-334)	111 (79-153)
Blood	14 (12-18)	12 (11-13)	13 (12-14)	14 (13-17)	13 (11-14)

It is evident from the results in Table I that the effects of nicotinic acid deficiency in the rat resemble in part those observed in other species. After 70 days on the deficient diet, there is little change in the nicotinic acid content of heart, lung, spleen, kidney, and blood. Only in liver, voluntary muscle, and brain are subnormal values found. The changes in liver and muscle are similar to those reported for the rat by Hundley (25) and those for other species. It is surprising, however, to find a certain dependence of the nicotinic acid level of brain on the dietary level of the vitamin. By contrast, in the dog and pig (14) normal brain levels are maintained in

spite of extreme deficiency. It appears that the major nicotinic acid stores in the rat are in the liver and muscle, when one considers not only the mass of tissue involved, but also its nicotinic acid content.

The administration of nicotinic acid completely prevents the changes in concentration of tissue vitamin which occur in animals on the basal ration alone. The values obtained are in general agreement with those found in animals reared on a stock ration which permits normal growth and breeding in this laboratory. With supplementary tryptophan essentially similar results are found. With tryptophan regarded as a biological precursor of nicotinic acid (26-28), the nature of its beneficial effect in maintenance of normal levels of tissue nicotinic acid is obvious. One finds levels in liver nicotinic acid which are above normal. Values as high as 334  $\gamma$  per gm. are well outside the normal range. Similar results obtained since the completion of the present investigation have been reported (29) and interpreted as supporting evidence of the synthesis of nicotinic acid in the liver. As judged by the strict maintenance of normal values in other tissues, one might surmise that the liver is the major site of synthesis.

Najjar *et al.* (30) have reported that N<sup>1</sup>-methylnicotinamide, a major metabolic end-product of nicotinic acid, possesses definite antiblacktongue activity, both as a preventive and as a therapeutic agent. This is in contrast to the findings of Teply *et al.* (31). In the present study this substance is inactive in rats at a daily level of 200  $\gamma$ . It does not prevent either growth retardation or depletion of the nicotinic acid stores in the liver and muscle that is observed on the basal ration. It would appear that this species is unable to demethylate N<sup>1</sup>-methylnicotinamide to nicotinic acid at a rate sufficient for good growth. In light of these results it is difficult to account for the lipotropic activity of N<sup>1</sup>-methylnicotinamide in rats on diets containing glycoeyamine, as reported by Najjar (32). If labile methyl groups are made available in a demethylation process, nicotinic acid *per se* cannot be considered an end-product, in view of the fact that the methylated derivative is inactive both as a preventive and as a therapeutic agent<sup>1</sup> in rats on the deficient basal ration.

#### DISCUSSION

The maintenance of the normal vitamin content of certain tissues in animals on diets deficient in this essential has been regarded as a necessary condition for the preservation of certain vital functions. A decrease is considered incompatible with life. In this respect the rat maintains normal nicotinic acid levels in the heart, lung, spleen, kidney, and blood, but allows

<sup>1</sup> 1.0 mg. of N<sup>1</sup>-methylnicotinamide was injected each day for 5 days into nicotinic acid-deficient rats. No alteration in the growth rate was observed during the next 14 days.

depletion of vitamin stores in muscle and liver, and, to a smaller extent, in brain. The depressed values must reflect a diminished capacity for metabolic functions requiring the presence of nicotinic acid as a coenzyme. However, the rat on inadequate diets makes a nutritional adaptation by reducing the food intake and thereby lessening the effects of nutritional stress. When this curtailment of food intake is prevented by forced feeding, acute deficiency states are obtained in a relatively short time without the complication of a generalized undernutrition. An excellent example of this has been reported recently by Spector in the production of tryptophan deficiency in the rat (33).

#### SUMMARY

The nicotinic acid content has been determined in some tissues of rats on diets containing corn, deficient in nicotinic acid. In the deficiency state normal values are maintained in the heart, lung, spleen, kidney, and blood. In the liver, muscle, and brain subnormal levels are obtained.

With supplementary nicotinic acid or tryptophan the depletion of the nicotinic acid stores is prevented. In this respect the beneficial action of the amino acid is interpreted in the light of the biosynthesis of nicotinic acid.

N<sup>1</sup>-Methylnicotinamide does not prevent either the growth retardation or the depletion of nicotinic acid stores in the liver and muscle that is observed in rats on the deficient diet.

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# THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

## VIII. CONDITIONS FOR OXALACETATE OXIDATION IN WHOLE HOMOGENATES\*

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(Received for publication, July 17, 1948)

In the development of the conditions for oxalacetate oxidation in animal tissues two sets of variables had to be studied. In the paper by Potter, LePage, and Klug (1) the homogenate was the chief variable. It was shown that isotonic KCl homogenates were superior to water homogenates, that the enzyme was stable in KCl homogenates at 0° for 6 hours or in the animal for 30 minutes post mortem, and that the enzyme was rapidly inactivated in the absence of substrate and cofactors at 38°. The present paper will report the results of studies in which the reaction mixture was varied in an attempt to define the optimum conditions for the oxidation, which are approximately the same as those previously used (1). The importance of this work lies in the fact that this reaction mixture can be used as a basis not only for studying the oxalacetate oxidation *per se* but also for studies on endergonic reactions which must be coupled to adenosine triphosphate (ATP). It must be emphasized, however, that the conditions described below are not necessarily the optimum for endergonic syntheses but are to be considered as a basis for further development of such reactions.

### EXPERIMENTAL

*Methods*—Oxygen uptake was taken as a measure of enzyme activity, and the measurements were made in a conventional Warburg apparatus at 38°. In addition, it was frequently desirable to determine the rate of substrate disappearance and of product formation. This was done by stopping the reaction in appropriate flasks at various intervals by adding 2 ml. of cold 17.5 per cent trichloroacetic acid. The precipitated proteins were then centrifuged in the cold, and aliquots of the supernatants were analyzed.

The homogenates were always prepared with 9 volumes of isotonic KCl

\* This work was aided in part by a grant from the Jonathan Bowman Fund for Cancer Research.

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per weight of tissue, with the precautions as to speed and cold previously indicated (1). Cold homogenates were added to cold reaction mixtures, which were then placed in the 38° bath. The final volume was always 3.0 ml. Readings were taken every 10 minutes, following a 10 minute equilibration period.

The analytical work herein reported involved mainly the disappearance of oxalacetate and the formation of citrate, although in experiments not reported determinations for  $\alpha$ -ketoglutarate, succinate, and malate were carried out and these substances were shown to be formed in the few reactions examined. Oxalacetate gives the same reaction with 2,4-dinitrophenylhydrazine that is given by pyruvate when the treatment with the reagent is continued for 20 minutes in the direct method of Friedemann and Haugen (2). Interference due to  $\alpha$ -ketoglutarate can be corrected by determining the optical density at two wave-lengths (480 and 580  $m\mu$ ), but in most of the work this was not necessary. Since oxalacetate and pyruvate give the same color density, the determination of keto acid disappearance reveals the conversion of oxalacetate and pyruvate into other compounds and does not show any interconversion that may occur between the two compounds. The primary product of the oxidative reaction that results in oxalacetic disappearance is not known, but the formation of citrate would constitute strong evidence that the Krebs condensation had occurred. This substance was determined by the pentabromoacetone method as modified by Natelson *et al.* (3), with single extractions suggested by Lardy (4). The method may be further simplified by carrying out the reactions and extractions in open 50 ml. graduated centrifuge tubes. The extractions can be completed in about 1 minute by means of a high speed stirrer in place of shaking. Aliquots containing 10 to 60  $\gamma$  of citric acid are suitable (3).

*Optimum Concentrations*—Table I summarizes a number of experiments in which varying concentrations of ATP, oxalacetate,  $MgCl_2$ , inorganic phosphate, and KCl were individually tested in the presence of the optimum concentrations of all the other reactants. Homogenates of rat kidney and rat liver were tested and gave approximately the same optima. Cytochrome *c* was present in all the flasks at a concentration of  $1.3 \times 10^{-5}$  M, although the beneficial effect was very slight and the compound could probably be omitted for most purposes. For example, in another set of experiments, the omission of the cytochrome decreased the oxygen uptake from 125 to 118 microliters per 20 minutes in the case of kidney and from 89 to 76 microliters in the case of liver.

All of the experiments in Table I were carried out with K salts instead of Na salts. In separate experiments it was found that the use of K salts produced a very marked stimulation in the case of kidney, while the effect

in the case of liver, though slight, was still significant. Thus with kidney homogenate, the average of two experiments done in triplicate was 126 microliters per 20 minutes with K salts and only 71 microliters with Na salts. With liver the values were 114 and 101 microliters respectively.

TABLE I

*Optimum Conditions for Oxalacetic Oxidase System in Liver and Kidney Homogenates*

The source of enzyme was rat tissue; 0.3 ml. of kidney or 0.5 ml. of liver as a 10 per cent homogenate in isotonic KCl was added to each flask. The data for each compound were obtained from two or more experiments in duplicate, with freshly prepared homogenates. Thus the variation in animals is the source of the variation in plateau values obtained with various compounds. Each compound was studied at varying concentrations and all other compounds were held constant and at the optimum values. In addition to materials in the table  $1.3 \times 10^{-5}$  M cytochrome *c* was present in each flask. The concentration of each component selected for the standard technique is indicated by bold-faced type. The measure of activity was arbitrarily taken as the microliters of oxygen taken up in the 20 minutes following the 10 minute equilibration period.

ATP		Oxalacetate		MgCl <sub>2</sub>		Phosphate		KCl*	
Molarity $\times 10^4$	O <sub>2</sub>	Molarity $\times 10^2$	O <sub>2</sub>	Molarity $\times 10^3$	O <sub>2</sub>	Molarity $\times 10^3$	O <sub>2</sub>	Molarity $\times 10^2$	O <sub>2</sub>
Kidney									
0	13	0	12	0	57	0	59	0	39
3.3	61	0.9	61	<b>3.3</b>	<b>132</b>	1.7	100	3.3	115
6.7	112	1.8	120	3.3	129	<b>3.3</b>	<b>103</b>	<b>6.7</b>	<b>130</b>
10.0	<b>124</b>	2.7	<b>122</b>	5.0	129	6.7	116	10.0	132
16.7	126	4.4	97	6.7	125	10.0	120		
						16.7	120		
Liver									
0	32	0	41	0	60	0	68	0	35
3.3	65	0.9	58	1.7	<b>82</b>	1.7	74	3.3	91
6.7	75	1.8	85	3.3	74	<b>3.3</b>	<b>71</b>	<b>6.7</b>	<b>81</b>
10.0	80	2.7	85	5.0	80	6.7	72	13.3	60
16.7	90	4.4	86	6.7	78	10.0	75	26.7	61
						16.7	80		

\* Does not include KCl in the homogenate.

The data in Table I were obtained with reaction mixtures of approximately pH 7.0. Earlier work was done at about pH 7.4. The effect of pH on the oxygen uptake is shown in Fig. 1. The optimum pH for the system is not a property of any one enzyme, but represents the resultant of the various optima of different enzymatic components of the system. This fact probably explains the difference between the pH curve for liver

compared to kidney, in which the ratio of the various enzymes involved is considerably different.

None of the data in Table I were obtained with mixtures that included DPN (diphosphopyridine nucleotide, coenzyme I). A large number of experiments were carried out on a series of ATP levels with and without added DPN. The DPN was of high purity (about 80 per cent), obtained

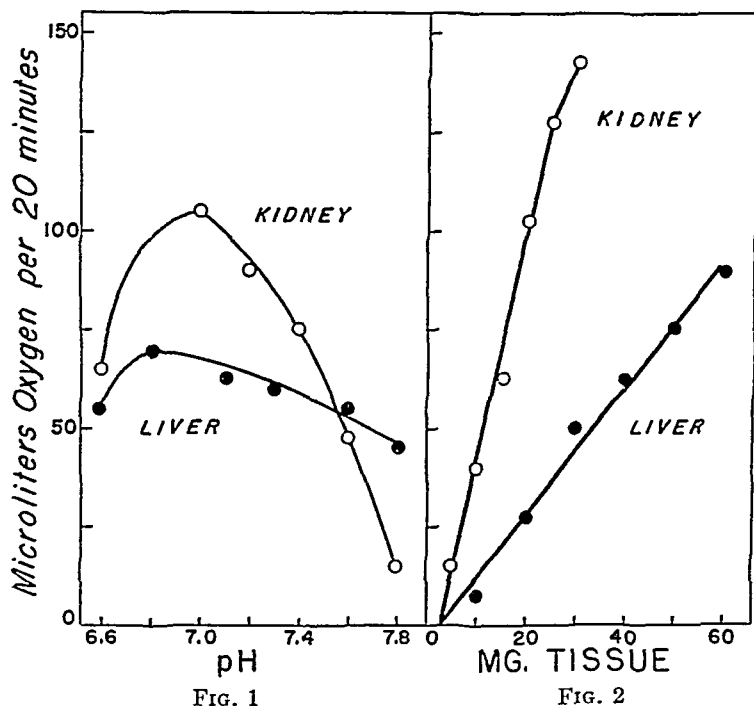


FIG. 1

FIG. 2

FIG. 1. Effect of pH on oxalacetate oxidation by isotonic KCl homogenates of rat kidney (30 mg.) or liver (50 mg.). The reaction mixture was as described in the text except that the phosphate buffer was increased to 0.033 M. The data are averages of two experiments in duplicate. The pH values were essentially constant during the experimental period.

FIG. 2. Effect of tissue concentration on rate of oxygen uptake in the oxalacetate system, as described in the text.

from Dr. G. A. LePage. The experiments showed that at low levels of ATP DPN produced marked stimulation, but, as the level of ATP was increased, the effect of DPN disappeared. It appears that DPN is not needed *per se* but that it can substitute for ATP, particularly in a system that contains a limiting amount of ATP. These results can be interpreted more readily in view of the recent results of Kornberg and Lindberg (5) who reported that an enzyme from kidney splits DPN to adenylic acid and

nicotinamide ribose phosphate. Since adenylic acid can be phosphorylated to ATP in the reaction mixture we employ, the effect of DPN is probably merely to serve as a source of ATP. The new DPN-splitting enzyme is not inhibited by nicotinamide (5), and we have been unable to obtain any beneficial effects with nicotinamide in the oxalacetate system in kidney and liver homogenates. In contrast to the kidney enzyme the DPNase in brain and in tumor is inhibited by nicotinamide, and no doubt each tissue will have to be studied individually to determine the effect of various additions on the system.

The data in Table I show that the addition of inorganic phosphate produces a marked stimulation and that high amounts of phosphate are not harmful. The higher levels of phosphate give much better buffering capacity, but, if changes in the phosphate concentration due to oxidative phosphorylation are to be studied, the experimental error becomes too high to permit accurate determination of small changes. The level of phosphate to be used is therefore a compromise between these two considerations and is generally at the high level when changes in the level are not being studied. The control of the pH of the reaction is one of the problems not fully solved. The initial addition of oxalacetate lowers the pH because the oxalacetic acid is only three-fourths neutralized. Large amounts of this oxalacetate can lower the pH to a damaging level. However, the decarboxylation to pyruvate results in the loss of 1 acid equivalent, and causes an alkaline shift if fully neutralized oxalacetate is used. High levels of tissue can tolerate high levels of the oxalacetate much better than lower levels of tissue because of the more rapid removal of acid equivalents.

An examination of the data in Table I shows that the following levels of reactants are satisfactory for both kidney and liver: 0.067 M KCl,  $3.3 \times 10^{-3}$  M  $\text{MgCl}_2$ ,  $3.3 \times 10^{-3}$  M potassium phosphate,  $1.3 \times 10^{-6}$  M cytochrome *c*,  $2.7 \times 10^{-3}$  M oxalacetate, and  $1 \times 10^{-3}$  M ATP (K salt).<sup>1</sup> The first four components can be combined into a stock solution of which 1.5 ml. are added per flask. The solution is prepared by adding 1.49 gm. of KCl, 0.203 gm. of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.136 gm. of  $\text{KH}_2\text{PO}_4$ , and 10 ml. of  $4 \times 10^{-4}$  M cytochrome *c* to 140 ml. of water, adjusting the pH to 7.0 with KOH, and making up to a final volume of 150 ml.

In addition to the 1.5 ml. of stock solution per flask, water is added to make the final volume 3.0 ml., followed by 0.3 ml. of 0.01 M ATP, 0.3 ml. of 0.0267 M fresh oxalacetate, and 0.2 to 0.7 ml. of 10 per cent KCl homoge-

<sup>1</sup> A commercial sample of sodium ATP (Rohm and Haas, Philadelphia) was found to be unsatisfactory in this system unless given further purification designed to remove traces of metallic inhibitors. The material as received yielded about 50 per cent activity compared with ATP prepared by Dr. G. A. LePage.

nate. All components were kept cold. The center wells were prepared before the oxalacetate addition, with 0.2 ml. of 2 N NaOH and filter paper. Table I shows that the addition of considerable amounts of extra KCl is without effect, and in general the variations in the amount of homogenate or addition of moderate amounts of extra substrates or inhibitors were compensated by merely adjusting the volume of the water added.<sup>2</sup>

*Proportionality*—One of the requirements of an assay system is that the amount of oxygen uptake is proportional to the amount of tissue added. The conditions described above permit oxygen uptakes within the range of convenient manometric measurement that are proportional to the amount of either kidney or liver homogenate, as shown in Fig. 2. With higher levels of oxalacetate such data are not obtained, and the deviation is especially marked at the lower levels of tissue. The data in Fig. 2 show a slight displacement to the right, which may be related to the oxalacetate effect or may be due to traces of some metal contaminant, although extreme precautions are taken to avoid metals.

*Nature of Reaction*—It is still not possible to accept the oxygen uptake data as an assay of a single enzyme. It appears that the oxalacetate is oxidized via the Krebs cycle, and in the case of liver and kidney appreciable amounts of citrate are formed. If the amounts of keto acid disappearing are plotted against time, it is seen that there is a rapid disappearance of keto acid, together with a rapid rise in citrate and malate. The reaction can also be analyzed by examining the effect of varying concentrations of the substrate upon substrate disappearance, oxygen uptake, and citrate formation, as shown in Fig. 3. Six different tissues, including two types of tumors,<sup>3</sup> were studied on this basis. The incubation time was 40 minutes and was chosen on the basis of the experiments referred to above. Although malate was not determined in this series of experiments, the data show that increasing amounts of oxalacetate result in a diminution of the oxygen taken up, while the amount of keto acid disappearing increases. This indicates that with increasing amounts of oxalacetate the reduction of

<sup>2</sup> The function of the KCl appears to be mainly the regulation of the tonicity of the solution. A number of experiments were done with sucrose in place of KCl in view of recent studies with this compound (6). Isotonic sucrose homogenates (8.5 per cent sucrose) were superior to 30 per cent sucrose homogenates, although the latter appear to preserve the mitochondria better (6). The optimum final sucrose concentration was between 2.5 and 5.5 per cent. In 4.5 per cent sucrose the reaction was independent of KCl concentration up to 6 mg. of KCl per ml. Apparently the requirement for K ion is met by the other K salts in the medium. Sucrose homogenates showed no advantage over KCl homogenates in the oxidation of oxalacetate, although other possible advantages are not ruled out.

<sup>3</sup> We are indebted to Mr. B. E. Kline for supplying us with rats bearing the Flexner-Jobling and the Walker No. 256 transplantable tumors.

oxalacetate to malate assumes increasing importance and a dismutation of oxalacetate to malate and citrate begins to supplant the oxidative conversion of oxalacetate to citrate. These results with extremely small quantities of rat tissue fortified with ATP are thus in harmony with the older data in the literature, in which minces of pigeon breast muscle were used by Krebs (see (7)). They are also compatible with the results of Kalnitsky (8), who has used high concentrations of oxalacetate in the study of citrate formation. It is interesting to note that negligible quantities of citrate were formed by brain and heart homogenates, although keto acid

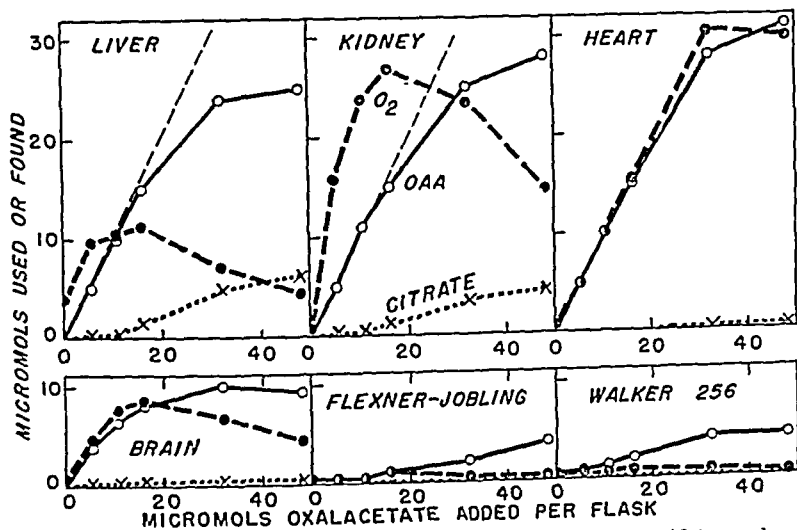


FIG. 3. Effect of oxalacetate concentration on oxygen uptake ( $O_2$ ), oxalacetate utilization (OAA), and citrate formation in the standard oxalacetate system but with 0.0167 M phosphate buffer. Tumors and normal tissues from rats (50 mg. per flask). The labels on the curves for kidney apply to the other tissues as well. The light dashed line in the case of liver and kidney gives the theoretical curve for 100 per cent removal of oxalacetate, and applies to all of the tissues.

disappeared and oxygen was taken up. The citrate formation in the charts is of course the resultant of the rates of formation and utilization. Thus the lack of citrate formation in the case of low levels of oxalacetate in kidney and liver is due to the fact that the citrate formation ceased when the oxalacetate was used up, and the accumulated citrate disappeared. The results with the tumor tissues support the earlier findings and show in addition that not only is the oxygen uptake very low but that the keto acid disappearance is very low also.

The above results suggest that the oxygen uptake during the initial part of the reaction may be a measure of that portion of the Krebs cycle which

is most closely connected with the oxidative phase of oxalacetate removal; hence our use of the term "oxalacetic oxidase." It is clear that the oxygen uptake very rapidly becomes the sum of several oxidative steps occurring in other parts of the cycle. Furthermore, the occurrence of alternative metabolic pathways is not excluded. For example, oxalacetate to pyruvate to acetate to acetoacetate could occur. However, the presence of oxalacetate tends to deflect this pathway into the Krebs cycle, as Lehninger has shown (9).

In view of the probability of other reactions in the cycle contributing to the oxygen uptake, it was of interest to determine the rates of oxidation for some of the compounds that might be involved. This was done with four

TABLE II  
*Relative Rates of Oxidation of Substrates*

The source of enzyme was rat tissue; 0.3 ml. of kidney, 0.5 ml. of liver, 0.4 ml. of heart, or 0.7 ml. of brain as a 10 per cent homogenate in isotonic KCl was added to each flask. The standard reaction mixture was used, except that phosphate was  $16.7 \times 10^{-3}$  M and the substrates were  $3.3 \times 10^{-3}$  M. The oxygen uptake in the 20 minutes following the 10 minute equilibration was taken as 100 for oxalacetate and the uptakes for other substrates are given relative to this value. The actual rates for oxalacetate are given as  $Q_{O_2}$ . The results are averages of duplicates on each of two animals generally.

	Kidney	$Q_{O_2}$	Liver	$Q_{O_2}$	Heart	$Q_{O_2}$	Brain	$Q_{O_2}$
Oxalacetate.....	100	52.0	100	16.1	100	25.5	100	15.0
Pyruvate.....	59		79		123		83	
Citrate .....	39		85		19		27	
$\alpha$ -Ketoglutarate.....	97		92		156		81	
Succinate.....	65		80		71		68	
Malate.....	55		75		46		41	
No substrate.....	8		44		2		32	

different tissues by use of pyruvate, citrate,  $\alpha$ -ketoglutarate, succinate, malate, no substrate, and oxalacetate for comparison. The results are shown in Table II. In each case the data are given in comparison with oxalacetate taken as 100 for the given tissue, but the absolute value for oxalacetate is also given. In heart homogenate, pyruvate was superior to oxalacetate, but in the other tissues it was not as actively oxidized. Similarly  $\alpha$ -ketoglutarate was rapidly utilized by heart. In contrast, citrate was very poorly utilized by heart, brain, and kidney in comparison with oxalacetate. Malate was also poorly utilized in comparison with oxalacetate. Thus there is little reason to believe that any member of the Krebs cycle could be substituted for oxalacetate in this system without substantially changing the results. In the data given in Table II, the

substrates were used at the same level as oxalacetate. In the assay, they would be present at much lower levels. The data in Table II do not represent the maximum rates for the other substrates by any means, while the rates for oxalacetate represent the maximum that we have been able to attain. Succinate could be oxidized much more rapidly by adding calcium and increasing the succinate concentration (10). Malate could be oxidized more rapidly by adding DPN and glutamate (11). The test system therefore applies chiefly to oxalacetate. It may be noted that a mixture of oxalacetate and pyruvate is no better than oxalacetate for experiments such as those described in this paper, although the mixture may offer some advantages in that larger amounts may be used with less alteration in the acid-base balance.<sup>4</sup>

### DISCUSSION

The conditions for maximum oxygen uptake in the oxalacetate system involve the use of a very low substrate concentration and the reaction soon fails for lack of substrate. The data suggest that the optimum substrate concentration is very low and in normal tissue *in situ* the oxalacetate concentration is probably no higher than this at the most. However, *in situ* the oxalacetate and pyruvate are continually being replenished and in addition the oxalacetate is in equilibrium with pyruvate and CO<sub>2</sub> by means of the Wood-Werkman reaction. In the Warburg flask, the use of the CO<sub>2</sub> absorbers in the center well cuts down the chances for this reaction to operate and limits the supply of oxalacetate to the original amount plus what can be formed from malate. Since the oxalacetate becomes depleted by decarboxylation, the studies *in vitro* are hampered by an inability to keep up the supply of oxalacetate. Possibly the reaction would benefit by the use of bicarbonate buffer and CO<sub>2</sub> in the gas phase (*cf.* Green *et al.* (12)).

The measurement of the oxygen uptake with the optimum level of oxalacetate for maximum rate is probably a fair measure of the oxidative component of the reactions involved in the formation of citrate, but there must be some reservations about any studies that involve oxygen uptake alone, and it is desirable to make analyses for as many products as possible. The complete lack of citrate accumulation in heart and brain raises the question of whether or not it was formed, and it would be of considerable interest to know whether acetate was formed to any extent in these tissues

<sup>4</sup> While the mixture of pyruvate and oxalacetate is no better than oxalacetate for short experiments with liver and kidney as reported in this paper, the mixture gives superior results with heart and brain, and, if pyruvate is present, other 4-carbon dicarboxylic acids give results that compare favorably with those obtained with oxalacetate only. With oxalacetate alone, an optimum level of oxalacetate and pyruvate appears to be rapidly attained in liver and kidney but not in heart or brain homogenates. (Added in proof.)



or in the tumor tissues. The observations on tumor tissues are of interest in view of the earlier findings of Elliott and Greig (13). Using tumor slices treated with malonate, they found no conversion of pyruvate to succinate, although some keto acid disappearance was noted, and acetic acid was tentatively identified.

#### SUMMARY

1. Optimum conditions for the study of the oxidation of oxalacetic acid by isotonic KCl homogenates of rat liver and kidney were described.
2. The final concentrations of reactants in the optimum system were 0.067 M KCl,  $3.3 \times 10^{-3}$  M  $MgCl_2$ ,  $3.3 \times 10^{-3}$  M potassium phosphate,  $1.3 \times 10^{-5}$  M cytochrome c,  $2.7 \times 10^{-3}$  M oxalacetate, and  $1 \times 10^{-3}$  M ATP. From 0.2 to 0.7 ml. of 10 per cent homogenate could be used, depending on the tissue being examined. The final volume was 3.0 ml.
3. The rate of oxygen uptake was proportional to the tissue concentration, within the range of convenient manometric measurement.
4. The optimum pH was about 7.0, and the use of potassium salts gave better results than when all sodium salts were used.
5. Analyses for citrate accumulation showed that this compound was formed when liver and kidney were used, but when brain, heart, and tumor were studied, the citrate accumulation was negligible. However, in brain and heart there were appreciable oxygen uptake and keto acid disappearance, while with the tumors (Flexner-Jobling and Walker No. 256 rat transplantable tumors) oxygen uptake and keto acid disappearance were both very low.

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# INHIBITION OF SUCCINIC DEHYDROGENASE BY OXALACETATE<sup>1</sup>

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(Received for publication, July 17, 1948)

Although the inhibition of succinic dehydrogenase by oxalacetate is a generally accepted fact, there is a paucity of data on the subject. In 1937 Das (1), using a modified Thunberg technique, reported that the enzyme was 50 per cent inhibited by  $2 \times 10^{-5}$  M oxalacetate when the succinate concentration was 0.025 M. In 1939 Potter (2) reported that the oxidation of succinate by liver and kidney homogenates was inhibited by cozymase (DPN). Keilin and Hartree (3) and Mann and Quastel (4) attributed this effect, no doubt correctly, to the formation of oxalacetate, although no data on the effect of oxalacetate were presented. The inhibitory effect of DPN upon the succinate system was later studied by Swingle, Axelrod, and Elvehjem (5) who also determined the effect of oxalacetate upon the succinic dehydrogenase system by measuring oxygen uptake. They reported that at succinate concentrations of 0.045 M oxalacetate produced 98, 65, and 22 per cent inhibition at concentrations of 10, 10, and  $5 \times 10^{-5}$  M. From the data given it is not possible to tell whether the inhibition was transitory, as will be shown below, or whether the experiments were of such short duration that the decreased inhibition was not revealed. Since we found that the inhibition declined with time, it is clear that the earlier experiments (1, 5) cannot be accepted as quantitative measures of the inhibition by oxalacetate. They do, however, establish the fact that this substance has a remarkable affinity for the succinic enzyme; remarkable because it appears to be at least 1000 times greater than the affinity of the normal substrate for the enzyme, and because oxalacetate has been assumed to be formed in the course of succinate oxidation. If the physiologically formed oxalacetate were as toxic to succinate oxidation as added oxalacetate, the inhibition would have profound regulatory effects upon oxidative metabolism. Such does not seem to be the case, however, although the reasons are as yet obscure.

\* This work has been supported in part by the Jonathan Bowman Fund for Cancer Research.

† Merck Postdoctoral Fellow in the Natural Sciences under the National Research Council.

## EXPERIMENTAL

Reactions were carried out in Warburg flasks at 38°. To each flask were added at 0°, in the order given, water to make the final volume 3.0 ml., 0.1 ml. of 1 M K phosphate buffer at pH 7.4, 0.1 ml. of  $4 \times 10^{-4}$  M cytochrome c, various amounts of 10 per cent water or KCl homogenates of rat tissues, 0.1 ml. of a solution containing 0.012 M  $\text{CaCl}_2$  and 0.012 M  $\text{AlCl}_3$ , and 0.3 ml. of 0.5 M sodium succinate essentially as in the succinoxidase assay system (6). In addition, various concentrations of freshly prepared oxalacetate and pyruvate were added in order to test their separate and combined effects upon the succinic system. The oxygen uptake was measured at 10 minute intervals after a 10 minute equilibration. Zero time was taken as the time when the flasks were placed in the 38° bath, which was immediately after the oxalacetate addition. Oxalacetate or pyruvate additions followed the succinate addition.

In reporting the data, the oxygen uptake for successive 10 minute periods is plotted against time at the mid-point of each time interval. Since in the succinate system the substrate is present in large excess in order to give the maximum velocity, this method of plotting the data gives the relative amount of the functioning succinic dehydrogenase throughout the course of the experiment.

Total keto acids were determined by the direct method of Friedemann and Haugen (7). In this procedure, oxalacetic and pyruvic acids give the same result when the incubation with the reagent is 20 minutes.

Later experiments were carried out with the succinate and oxalacetate added to the oxalacetate oxidizing system, which contains no Ca or Al, and has Mg and adenosine triphosphate (ATP) added (8). Under the conditions described for the succinoxidase system (6) the homogenate will not oxidize malate, oxalacetate, or pyruvate, and oxidizes succinate quantitatively to fumarate and malate.<sup>1</sup>

*Inhibition by Oxalacetate*—When succinate is added to the succinoxidase system, the rate slowly declines in a linear fashion, as previously described (9) and as shown in the top curve of Fig. 1. From the initial rates of five similar experiments with variable amounts of succinate the Michaelis-Menten constant (10) was determined for this system and the average value of 0.006 mole per liter was obtained for subsequent calculations. When various amounts of 0.001 M oxalacetate were added to the standard succinate system, the oxidation was strongly inhibited, but with the passage of time the inhibition diminished, so that the rate of the oxidation progressively increased and approached the declining control curve for succinate alone (Fig. 1). Analysis of the flask contents at the end of the experiment showed that the total keto acid was the same as at the beginning.

<sup>1</sup> Unpublished experiments.

In view of the fact that neither oxalacetic nor pyruvic acid is oxidized in this system plus the known fact that oxalacetate is both spontaneously and enzymatically decarboxylated to pyruvate, it is concluded that the release from inhibition is due to the decarboxylation of the oxalacetate to pyruvate.

It was found that oxalacetate produced 50 per cent inhibition at a final concentration of approximately  $2 \times 10^{-5}$  M based on the initial inhibition. At concentrations at which oxalacetate produces 50 to 100 per cent inhibition, pyruvate is without effect; so that conversion of oxalacetate to

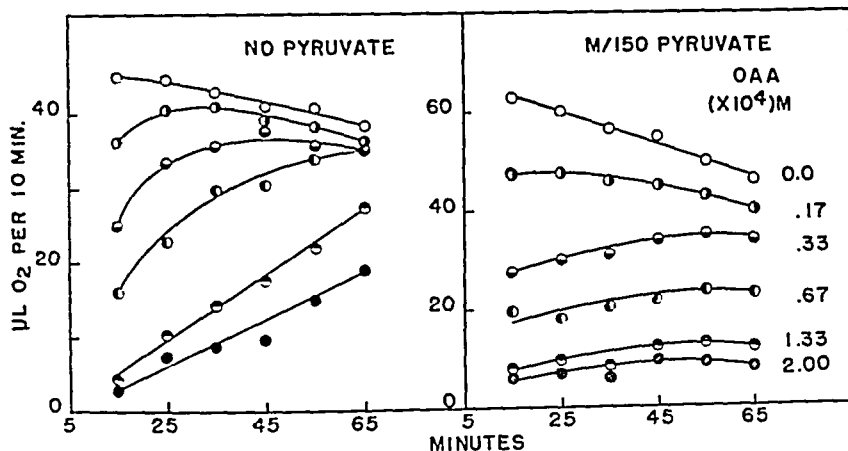


FIG. 1

FIG. 2

FIG. 1. Transitory inhibition of the succinoxidase system by various amounts of oxalacetate. Succinoxidase assay system described in the text, with 0.2 ml. of a 10 per cent water homogenate of rat liver per flask. Concentrations of oxalacetate as in Fig. 2, for the corresponding symbols.

FIG. 2. Stabilization of the inhibition of the succinoxidase system by oxalacetate (OAA) when pyruvate is present. Succinoxidase system as in Fig. 1. Oxalacetate concentrations refer to the final molarity.

pyruvate would account for the release from the inhibition. Final concentrations of 0.1 M pyruvate or  $\alpha$ -ketoglutarate gave 50 per cent inhibition, while malate and aspartate at similar concentrations had no effect. The effect of oxalacetate is unique in our experience with forty or 50 inhibitors of the succinic system (9), since no other inhibitor gave an immediate inhibition that decreased with time. Previous inhibitors have either acted at once and maintained the inhibition, like malonate, or have given progressively greater inhibition, like quinone. The behavior of oxalacetate, as shown in Fig. 1, indicates that oxalacetate forms a dissociable complex with succinic dehydrogenase, as do succinate and malonate.

Since both Ca and Al have been shown to increase the spontaneous

decarboxylation of oxalacetate (11), experiments with either or both of those ions omitted were carried out, but the results were not affected very much and are complicated by the possibility of oxalacetate formation from succinate. On the other hand, when the amount of homogenate was varied, the rate at which the oxalacetate effect wore off was greatly increased, as will be shown later. This effect is attributed to the enzymatic decarboxylation of oxalacetate to pyruvate. Furthermore, the rate of release from oxalacetate inhibition was different in different tissues, which fell in the following order of decreasing rates: kidney, liver, heart, brain, and tumor (Walker No. 256 rat carcinoma). This property is probably a reflection of oxalacetate decarboxylation but was not studied extensively, and most of the experiments were carried out with rat liver.

*Inhibition by Oxalacetate Plus Pyruvate*—Since moderate concentrations of pyruvate do not inhibit succinate oxidation, it is possible to study the effect of pyruvate on the oxalacetate inhibition. It appears that at least with the low concentrations of oxalacetate used in these experiments pyruvate inhibits oxalacetate decarboxylation. In experiments in which various levels of oxalacetate were studied in the presence of  $M/150$  pyruvate, the inhibition of the succinate system remained relatively constant throughout the experiment, showing a very slow rate of release (Fig. 2). These curves are in marked contrast to those in Fig. 1. The testing of the inhibition of the succinate system with and without pyruvate appears to be a highly specific test for oxalacetate, and is applicable to quantities of 10 to 50  $\gamma$ . These amounts are far below the quantity required for methods that depend on  $CO_2$  evolution. The test may be of some value when it is necessary to distinguish between oxalacetate and pyruvate.

*Inhibition by Oxalacetate Produced from Succinate*—In the assay method which has been proposed for the succinoxidase system (6), calcium and aluminum ions were added because of their demonstrated stimulatory effects, which were believed to be indirect. Swingle, Axelrod, and Elvehjem (5) had provided substantial evidence favoring the view that calcium is stimulatory because it accelerates the breakdown of DPN contained in the homogenates. It was postulated that the DPN in the homogenates would be sufficient, in the absence of calcium ions, to yield inhibitory amounts of oxalacetate. However, they did not determine the keto acids formed in the presence or absence of calcium in the succinoxidase system. Such a determination would test directly for the occurrence of the malate to oxalacetate step. We have now tested their idea by determining the effect of various additions to the succinoxidase system in terms of keto acid formed and oxygen taken up. The results of three such experiments with rat kidney homogenates are shown in Fig. 3. The same experiment was also carried out with liver with similar though less striking

results.<sup>2</sup> The decreased oxygen uptake is clearly correlated with the formation of keto acid, which in view of the results in Figs. 1 and 2 must be a mixture of oxalacetic and pyruvic acids. For example 0.3 micro-mole of keto acid per flask is  $1 \times 10^{-4}$  M, which if 100 per cent oxalacetate would produce much more inhibition (Fig. 2) than was actually observed (Fig. 3).

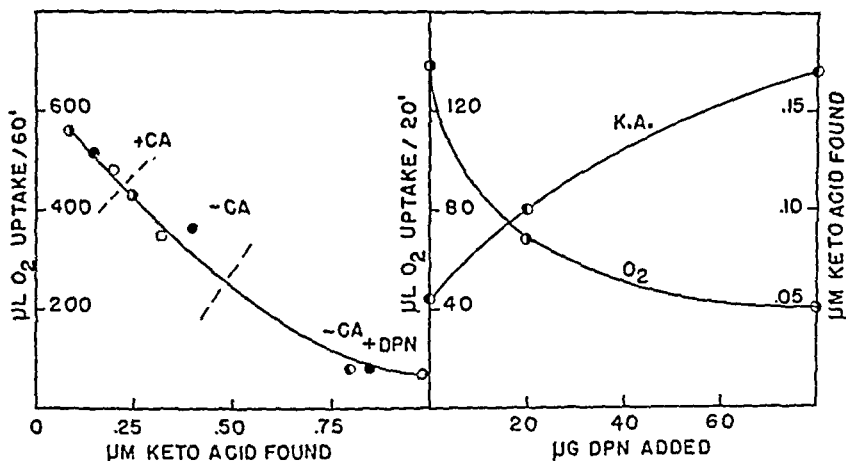


FIG. 3

FIG. 4

FIG. 3. Plot of oxygen uptake against keto acid formed in the succinoxidase system under various conditions to show that the formation of keto acid *decreases* the oxygen uptake. Experiments with three different rats represented by three different symbols. Each experiment was performed with 0.2 ml. of a 10 per cent cold isotonic KCl homogenate of rat kidney. Part of the curve, marked +Ca, represents conditions of standard succinoxidase assay (except for homogenate), the middle part of the curve, marked -Ca, represents the effect of omitting calcium, and the lower part of the curve, marked -Ca, +DPN, represents the effect of omitting calcium and adding DPN (approximately 400 γ).

FIG. 4. Effect of adding various amounts of DPN to the succinoxidase system in terms of both oxygen uptake and keto acid formation. Standard succinoxidase system with 0.1 ml. of a 10 per cent water homogenate of rat kidney.

The oxygen uptake was quite constant during the 60 minute period in the flasks in which Ca was omitted or DPN was added (Fig. 3), indicating that a steady state level of oxalacetate was rapidly reached. In other experiments with periodic sampling, the total keto acid increased with time. There was thus a continual production of oxalacetate that was maintained at a constant level by concomitant conversion to pyruvate.

<sup>2</sup> The data in Fig. 3 were obtained with KCl homogenates, but water homogenates gave almost identical results. With liver, however, the water homogenate did not give the same quantitative result that was obtained with the KCl homogenate. In all cases, decreased oxygen uptake was correlated with increased total keto acid.

The succinoxidase system is very sensitive to the presence of DPN because of the resultant oxalacetate that is formed: increased amounts of DPN (20 to 80  $\gamma$ ) give increased amounts of keto acid and decreased oxygen uptake (Fig. 4).

These results emphasize the importance of observing the recommended conditions for the succinoxidase assay system (6). Together with the results in Fig. 1, they show that the succinate to fumarate step can be isolated, and that, if small amounts of oxalacetate are formed initially, the resulting inhibition will disappear. Thus the maximum rate observed is the best measure of the succinoxidase system. In practice, the initial readings are seldom low.

*Prevention of Oxalacetate Effect*—In view of the failure to obtain maximum succinate oxidation in homogenates in the absence of calcium, it was always difficult to explain how Rosenthal (12) obtained the same rates of

TABLE I

*Prevention by ATP of Inhibition of Succinoxidase by Oxalacetate*

Oxalacetate system as described by Potter, Pardee, and Lyle (8), with 0.2 ml. of a 10 per cent isotonic KCl homogenate of rat kidney plus 0.1 ml. of 0.5 M succinate, and, where indicated, calcium and aluminum as in the succinoxidase system described in the text. Oxygen uptake, microliters in 20 minutes; all measurements in duplicate; volume, 3.0 ml.

Reaction conditions	Oxalacetate added	
	None	0.00267 M
With 0.001 M ATP.....	117.5	132.6
ATP omitted.....	81.3	5.0
With ATP + calcium and aluminum.....	57.1	0

oxidation in rat liver slices that we obtained with homogenates. His results and the question of whether the oxalacetate concentration is a physiological regulator of succinate oxidation led us to test the effect of oxalacetate on succinate oxidation in a homogenate system that we regard as "physiological;" namely, the isotonic KCl homogenate, fortified with ATP and Mg (8). To our surprise, relatively large amounts of oxalacetate failed to inhibit succinate oxidation. In Table I it is shown that with the reaction mixture and the amount of oxalacetate that is optimum for the oxalacetate oxidation (8) oxidation proceeds vigorously when succinate is present with or without oxalacetate (132.6 and 117.5 microliters of  $O_2$ ). On the other hand, when ATP is either omitted or added in the presence of calcium ions, which accelerate the dephosphorylation of ATP, the presence of oxalacetate prevents the oxidation of succinate. Thus oxalacetate inhibits succinate oxidation in the absence of ATP but not in its presence.

In other experiments it was possible to show that in a reaction mixture containing oxalacetate, ATP, and succinate delayed additions of calcium ions produced almost immediate inhibition of the succinoxidase system, the degree of inhibition depending upon the time of calcium addition. It was as if the oxalacetate were in a form that would not inhibit the enzyme and the addition of the calcium caused the oxalacetate to resume the inhibitory form. It may be emphasized that calcium *per se* does not inhibit the succinoxidase system. The question arises as to whether in the presence of ATP the oxalacetate might inhibit succinate oxidation but by being oxidized itself might obscure the succinate oxidation. In Table II various amounts of oxalacetate were added to a system containing ATP, with and without succinate. In the absence of succinate, the data show that the lower levels of oxalacetate were incapable of giving a rate of oxygen uptake that would account for the amount observed when succinate was also

TABLE II

*Oxidation of Succinate in Presence of Oxalacetate and ATP*

Oxalacetate system as described by Potter, Pardee, and Lyle (8), with 0.3 ml. of a 10 per cent isotonic KCl homogenate of rat kidney plus 0.3 ml. of 0.01 M ATP with various amounts of exactly neutralized oxalacetic acid with and without succinate. Volume, 3.0 ml.; oxygen uptake, microliters in 20 minutes

Succinate	Final oxalacetate concentration ( $\times 10^4$ M)					
	0	3.3	6.7	13.3	26.7	53.3
Present	213	215	205.8	210.5	196.6	197.6
Absent	6.9	44.7	65.5	84.6	109.5	84.4

present. It must be concluded, therefore, that these levels of oxalacetate do not inhibit succinoxidase in the presence of ATP. Yet Figs. 1 and 2 showed that one-tenth as much oxalacetate inhibited the succinoxidase system in the absence of ATP.

## DISCUSSION

*Inhibition of Succinoxidase by Oxalacetate*—The results can be accounted for by assuming that oxalacetate competitively inhibits the oxidation of succinate according to the equation

$$v = \frac{SV}{K_s(1 + (X/K_i)) + S}$$

where  $v$  is the rate of oxygen uptake,  $V$  is a constant,  $S$  is succinate concentration,  $K_s$  is the Michaelis-Menten constant,  $X$  is the oxalacetate (inhibitor) concentration, and  $K_i$  is the dissociation constant of oxalacetate and



enzyme. Let  $H$  be the rate of  $O_2$  uptake in the absence of  $X$ , and write the equation in the form

$$\frac{K_s X}{K_i(S + K_s)} = \frac{H}{v} - 1$$

If  $S$  is essentially constant during the measurements, as it is in these experiments,  $X$  is proportional to  $(H/v) - 1$ . If it is assumed that the rate of breakdown of oxalacetate is first order,  $\log X_0/X = kt$ , where  $X_0$  is the concentration of  $X$  at zero time, a plot of  $\log ((H/v) - 1)$  versus  $t$  should give a straight line of intercept at zero time,

$$\log \frac{K_s X_0}{K_i(S + K_s)}$$

and slope,  $-k$ .

Data from an experiment similar to that shown in Fig. 1, but with variable homogenate and constant oxalacetate, were plotted in this way and gave the results shown in Fig. 5. The intercept at zero time, which is proportional to the oxalacetate added (see below), is the same for all three concentrations of homogenate, showing that the initial inhibition is independent of the amount of homogenate. In the lower left corner of Fig. 5 the slopes of the three lines are plotted against the amount of homogenate and a straight line is obtained, showing that in addition to the slow spontaneous decarboxylation there was a decarboxylation that was proportional to the homogenate concentration.

With the same method of plotting the data, but with constant homogenate and variable oxalacetate, a series of lines having the same slope but different intercepts is obtained (Fig. 6). When the antilogs of the intercepts were plotted against the concentration of oxalacetate, the result was as shown in Fig. 7. The graph should be a straight line through the origin and the slight displacement to the right may be due to a small spontaneous decomposition before the beginning of the experiment.

The value for  $K_i$  can be calculated from Fig. 7, and is found to be  $1.5 \times 10^{-6}$  M. Thus oxalacetate is a much more powerful inhibitor than malonate, for which the constant is  $10^{-4}$  M, calculated from Potter and DuBois (9).

*ATP Effect*—Although oxalacetate is a powerful inhibitor of the succinoxidase system as shown in Figs. 1 and 2, it appears that this effect can be prevented by the presence of ATP. Whether the ATP acts by shielding the succinoxidase directly or indirectly or whether the ATP converts the oxalacetate to a form that cannot combine with the succinic dehydrogenase cannot be decided at this time. It has been suggested by various investigators that a phosphooxalacetate may exist, but only indirect evidence

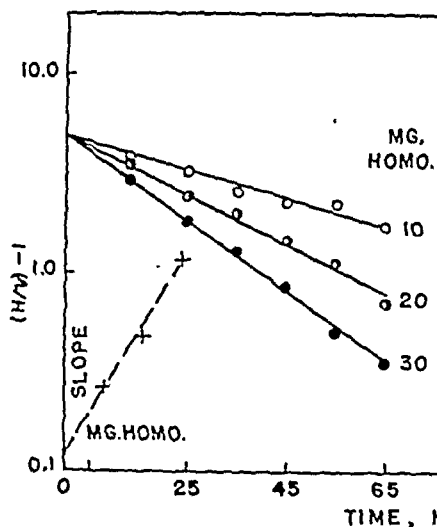


FIG. 5

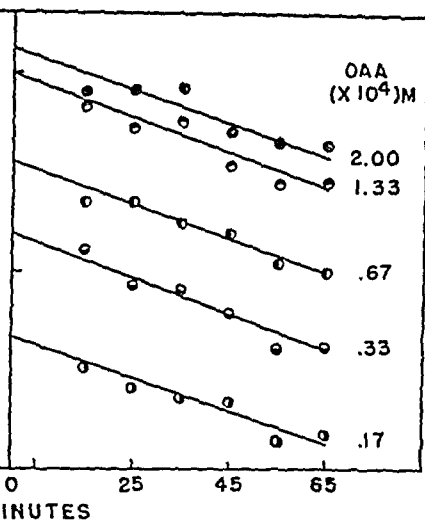


FIG. 6

FIG. 5. Log of  $(H/v)-1$  plotted against the time of reaction for three amounts of liver homogenate as indicated. Succinoxidase system as in the text. Initial concentration of oxalacetate constant at  $6.7 \times 10^{-5} M$ . The inset in the lower left corner is a plot of the slopes of the three curves against the amount of tissue used.

FIG. 6. Log of  $(H/v)-1$  plotted against the time of reaction with homogenate constant and oxalacetate variable; data from Fig. 1.

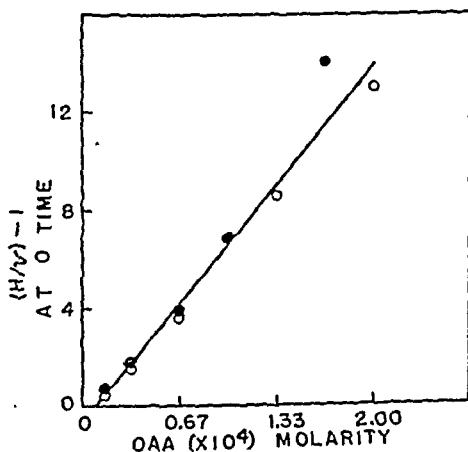


FIG. 7. Plot of values of  $(H/v)-1$  at zero time against concentration of oxalacetate added. O, data from Fig. 6; ●, from a similar experiment.

as been obtained (cf. Lichstein and Umbreit (13) p. 335, and Lardy *et al.* (14)). The experimental conditions described above may facilitate the attempts to study this problem.

The results with oxalacetate are of considerable interest in connection with an attractive suggestion by Pauling ((15) p. 58) that the surface configuration of the enzyme is complementary to the activated substrate rather than to the substrate itself. He proposed to test this postulate by searching for inhibitors that have a greater affinity for the enzyme than have the substrate molecules themselves. The succinic dehydrogenase system is one of the few that presents this opportunity; both malonate and oxalacetate have greater affinities than succinate for the enzyme. If ATP could convert oxalacetate to phosphoenol-oxalacetate, the configuration would be altered considerably, and no doubt to an extent that would prevent it from combining with the points having succinate affinity. This would seem to be the simplest explanation for the data at hand, but further experiments will have to be devised before it can be accepted.

#### SUMMARY

1. Oxalacetate inhibits the enzymatic oxidation of succinate. The dissociation constant of oxalacetate and enzyme is  $1.5 \times 10^{-6}$  M.
2. The oxalacetate gradually breaks down to pyruvate and the inhibition disappears.
3. Pyruvate decreases the rate of breakdown of oxalacetate, presumably by inhibiting the oxalacetic decarboxylase.
4. A mechanism is given based on competitive inhibition, and the first order rate of decarboxylation of oxalacetate.
5. ATP prevents the inhibition of the succinoxidase by oxalacetate while the addition of calcium ions to an ATP-protected system restores the inhibition.

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# XANTHINE OXIDASE AND TYROSINASE IN THE LIVERS OF CHICKS RECEIVING GRADED LEVELS OF DIETARY PTEROYLGLUTAMIC ACID\*

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(Received for publication, July 26, 1948)

Evidence indicating a relationship between pteroylglutamic acid (PGA) and porphyrin metabolism has been presented previously from this laboratory (1). While this supposed relationship was being investigated, it was noted that in *in vitro* experiments conducted by the procedures of Schreus and Carrié (2) liver homogenate from PGA-deficient rats rapidly converted hemoglobin to methemoglobin, and that this conversion was inhibited by PGA. Upon subsequent incubation the PGA-containing mixtures produced consistently a somewhat smaller amount of porphyrin than did the controls not containing PGA.

Rodney, Swendseid, and Swanson (3) have reported that liver homogenate from PGA-deficient rats fed sulfonamides does not oxidize tyrosine at a normal rate. They also reported that added PGA increased the rate of oxidation under the conditions of their experiments. In somewhat similar experiments, with liver from PGA-deficient chicks, we have been unable to show any enhancement of tyrosine oxidation; instead, when PGA was added, we found a slight but consistent inhibition of oxygen uptake as compared with controls either in the presence or absence of tyrosine.

The recent publication by Kalckar of experiments indicating an inhibitory effect on xanthine oxidase by PGA (4) or by one of its breakdown products (5) appeared to offer an explanation for both of our observations discussed above. It is well known that methemoglobin may be formed during the oxidation of substrates of xanthine oxidase if hemoglobin is present. Presumably there would be enough of the precursors of endogenous uric acid present in the chick liver experiments to account for a small uptake of oxygen which might be inhibited by PGA. However, it has been found in later experiments<sup>1</sup> that xanthine oxidase may be only one of several flavin-con-

\* Research paper No. 877, Journal Series, University of Arkansas. This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service, and a grant from the Williams-Waterman Fund of the Research Corporation.

<sup>1</sup> Totter, J. R., Martindale, W. E., and Keith, C. K., unpublished experiments.

taining enzymes which are inhibited by PGA or by its metabolic products. The later observation of Kalekar (5) that specially purified PGA exhibits but little inhibitory effect on xanthine oxidase makes it of importance to determine whether the *in vivo* action of the vitamin is related at all to this enzyme.

The experiments described in this publication were designed to determine whether the liver xanthine oxidase of chicks is affected by dietary PGA. There are also presented here data on the oxygen uptake of liver homogenate from PGA-deficient chicks as affected by tyrosine and added PGA.

#### EXPERIMENTAL

White Leghorn chicks were obtained from a commercial hatchery and were placed on the experimental diets at approximately 2 days of age. They were housed in metal battery brooders and were given the experimental diets and water *ad libitum*. The diet was similar to that recently recommended for use in chick assays for the vitamin M group (6). It consisted of vitamin-free casein (Labco) 25 gm., gelatin 10 gm., corn-starch 52.4 gm., cellulose 3 gm., hydrogenated vegetable oil 3 gm., cod liver oil 1.3 gm., salts 5 gm. (7), L-cystine 0.3 gm.,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  0.1 gm., choline chloride 0.2 gm., thiamine chloride 0.4 mg., riboflavin 0.8 mg., pyridoxine hydrochloride 0.6 mg., 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine lactone<sup>2</sup> 0.05 mg., calcium pantothenate 1.1 mg., nicotinic acid 2.0 mg., *i*-inositol 5.0 mg., *p*-aminobenzoic acid 15.0 mg., 2-methylnaphthoquinone 0.005 mg.,  $\alpha$ -tocopherol 24.0 mg., and biotin<sup>2</sup> 0.01 mg.

All chicks were weighed weekly for 4 weeks. In the first series, groups of chicks received synthetic PGA<sup>3</sup> incorporated in the diet to the extent of 0, 5, 10, and 200  $\gamma$  per 100 gm. of diet. Livers from these chicks were used for the tyrosinase determinations. Except for hematocrit determinations, no extensive hematological observations were made on the groups in this first series.

Xanthine oxidase was determined on the livers from a second series of chicks receiving 0, 5, 10, 20, 40, 80, 200, and 1000  $\gamma$  of PGA per 100 gm. of diet. This series was also controlled by including a group which received a commercial chick starter diet<sup>4</sup> containing 1.75  $\gamma$  of PGA per gm. by *Streptococcus faecalis* assay. In this second series, hemoglobin, hematocrit, total blood cell, and differential counts were made on each chick at the end of 4 weeks on the experiment. The chicks were then sacrificed and the organs removed for weighing. The livers were suspended in 5 volumes of phosphate buffer of pH 7.4 and homogenized in a Waring blender.

<sup>2</sup> Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

<sup>3</sup> Kindly supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

<sup>4</sup> Purina Startena.

The oxygen uptake of 1.0 ml. of 1:8 liver homogenate was measured at pH 7.5 in the absence of tyrosine and in the presence of 0.5 mg. of tyrosine, with the Warburg instrument. The total volume was 3.0 ml.

For the xanthine oxidase experiments 5 ml. of the liver mince from each chick were diluted to 25 ml. with phosphate buffer of pH 7.4. 2 ml. of the resulting mixture and 3 ml. of buffer were incubated with 5 ml. of a solution containing 5 mg. of xanthine in 0.01 N sodium hydroxide. A control with buffer instead of the xanthine solution was also set up. 1 ml. aliquots of these solutions taken immediately after mixing, and again after standing 1 hour at 32°, were treated with 1 ml. of 10 per cent sodium tungstate, 1 ml. of  $\frac{2}{3}$  N sulfuric acid, and 7 ml. of water, and filtered. Uric acid was determined on 2 ml. aliquots of the filtrates by Brown's procedure (8). The xanthine oxidase activity was expressed as mg. of uric acid produced per hour per gm. of wet weight of liver.

#### RESULTS AND DISCUSSION

The average weights for the chicks in the second series are presented in Fig. 1. The hematological values were in close agreement with values obtained in an extensive series of experiments by Campbell and coworkers (9). The average weights and the hematocrit values obtained in our experiments, conducted at different seasons, were also in satisfactory agreement. More detailed observations on the blood and bone marrow of the chicks on these experiments will appear elsewhere.

Attention should be called to the ratio of the liver weight to body weights presented in Fig. 2. A large increase in the ratio occurs with diminishing intake of PGA. Similarly the deficient chicks had an obviously enlarged gallbladder as compared with positive controls. The gallbladder weights of eight negative control chicks averaged 0.77 gm., while those of eight chicks in the 40  $\gamma$  of PGA group averaged 0.25 gm. The chicks on the commercial diet had a liver weight-body weight ratio of 0.0254, much lower than those receiving PGA far in excess of minimum requirements.

A typical tyrosinase experiment is given in Fig. 3. It may be seen that there is a decrease in oxygen consumption of the livers from deficient chicks in the presence of added PGA. This decrease is small; however, it was repeatedly observed in determinations made on livers of chicks receiving 0, 5, or 10  $\gamma$  of PGA. No consistent effect of the dietary intake of PGA on the tyrosinase content of the livers was obtained. Also, there was no appreciable effect on tyrosinase found when PGA was added to the incubating mixtures as shown in Fig. 3. These results differ from those of Rodney, Swendseid, and Swanson (3) who made their observations on livers from sulfonamide-treated rats. The reasons for the differences in outcome between the two experiments are not apparent. Since there is obviously a relation between

tyrosine metabolism and the action of PGA and of the antipernicious anemia principle (10, 11), caution in interpreting the results is indicated.

Results of the xanthine oxidase experiments are given in Fig. 4. An inverse relationship between PGA intake and xanthine oxidase is clearly shown. When the large liver weight-body weight ratio is taken into consideration, it may be seen that the deficient chicks possess an extremely high total oxidase activity.

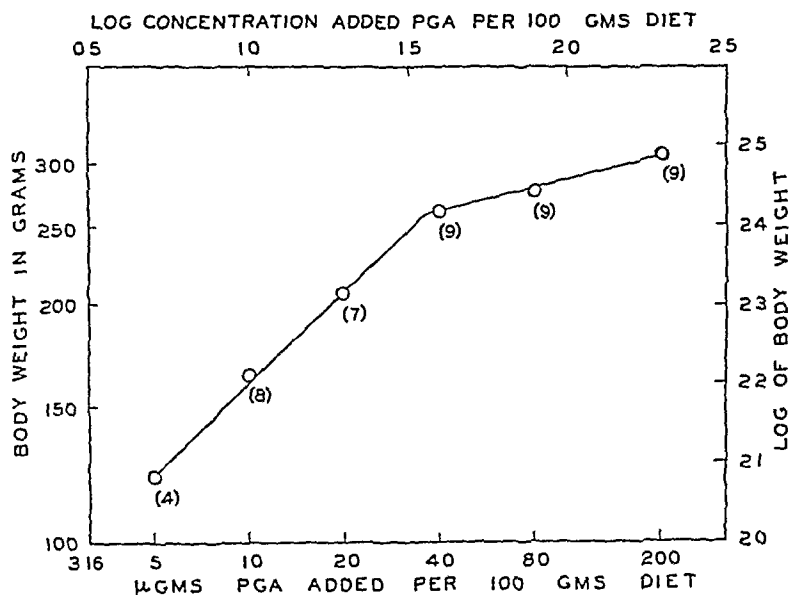


FIG. 1. The relation of the body weight of chicks to the pteroylglutamic acid content of the diet. The figures in parentheses are the number of chicks which survived the experimental period of 4 weeks. Initially the groups consisted of twenty, twelve, twelve, ten, ten, ten, nine, eight, and eight chicks for the 0, 5, 10, 20, 40, 80, 200, and 1000  $\gamma$ , and commercial starter diet, respectively. The final average weight of the fourteen surviving negative control chicks was 114 gm., that for the eight receiving 1000  $\gamma$  of PGA 247 gm, while the eight chicks on the commercial diet weighed an average of 286 gm. per chick.

Tests of the PGA used in our experimental diets indicate that it contains the impurity (5) which is responsible for the *in vitro* inhibition of xanthine oxidase. It may be argued that the impurity is responsible for the depression of the enzyme activity in the chick experiments. If so, the substance is of nutritional significance itself, and may occur in natural diets, since the xanthine oxidase activity of the livers from chicks on the commercial starter diet (0.94 mg. of uric acid per gm. per hour) was much lower than the lowest values obtainable with the supplemented purified diet. It is possible that additional dietary factors are present in the commercial diet which are necessary to maintain the low level of liver xanthine oxidase exhibited by the

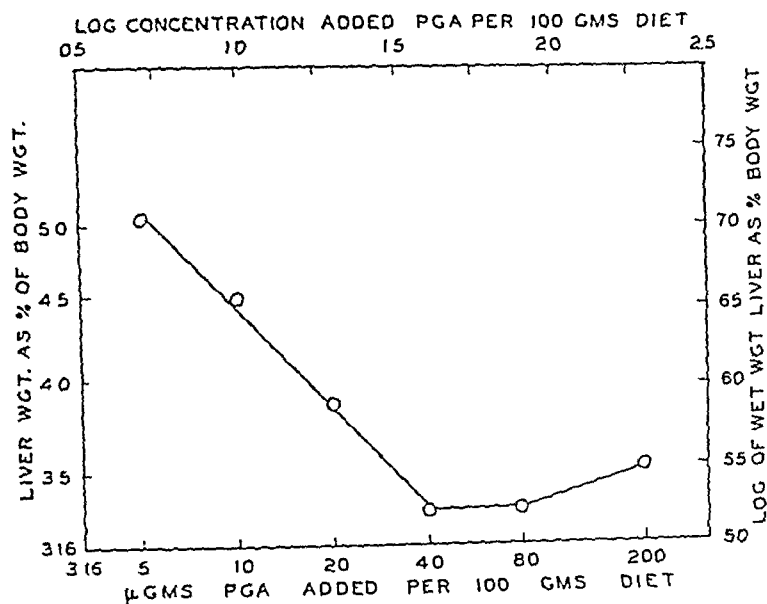


FIG. 2. The relative liver weight of chicks as affected by the PGA content of the diet. The numbers of chicks are the same as in Fig. 1. The fourteen negative control chick liver weights averaged 5.40 per cent, the eight of the 1000  $\gamma$  group 2.96 per cent, and the eight of the commercial diet group 2.54 per cent of their body weights.

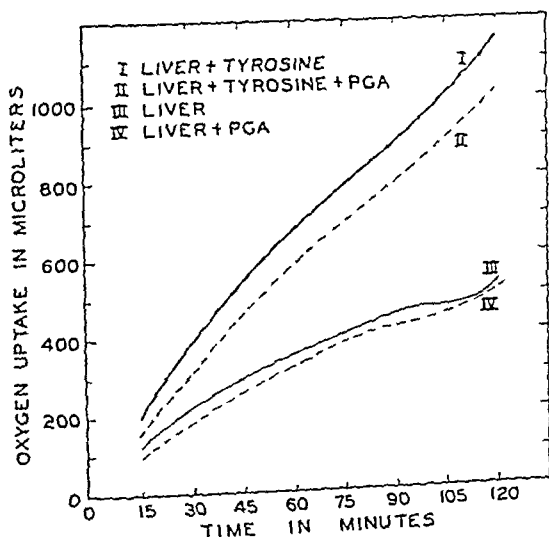


FIG. 3. The oxygen uptake of liver homogenate from PGA-deficient chicks in the presence and absence of added tyrosine. The abscissa gives the oxygen uptake in microliters per gm. of wet weight of liver. The liver used was a composite sample from three chicks.



chicks on such a diet. This phase of the problem is being investigated further.

The contribution which an excessively rapid rate of purine oxidation may make to the breakdown of hemoglobin or hemin-containing enzymes *in vivo* is unknown but may be of importance. The breakdown of hemoglobin is said to be brought about by hydrogen peroxide (12) and this compound is produced by the action of xanthine oxidase.

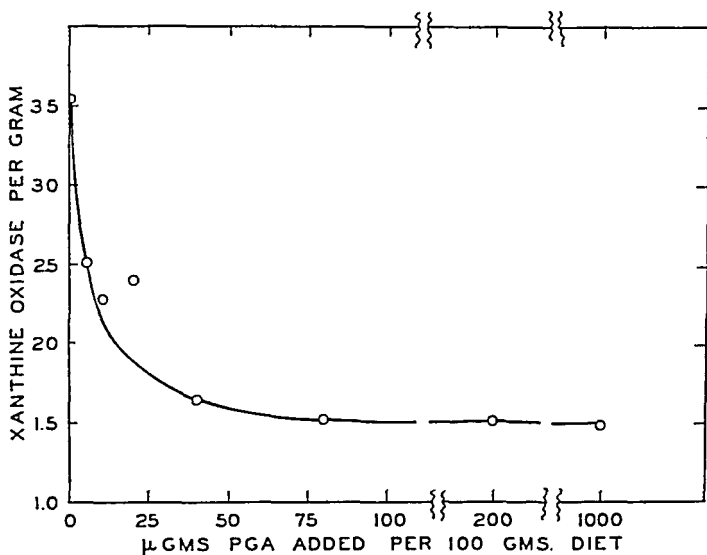


FIG. 4. The influence of PGA intake on chick liver xanthine oxidase. The abscissa gives the relative xanthine oxidase calculated as the mg. of uric acid produced from xanthine per gm. of wet weight of liver per hour. The number of determinations averaged for each point is the same as the number of chicks given in Fig. 1. The average xanthine oxidase for the fourteen negative control chicks was 3.55, while the eight on the commercial diet averaged 0.94.

It appears likely that other flavin-containing enzymes which catalyze the production of hydrogen peroxide as an end-product may also be involved in PGA deficiency and in the syndrome of pernicious anemia. Daft (13) and others (14) have presented results which may be interpreted as suggestive that L-amino acid oxidase activity is depressed by PGA. The recent findings of Woodruff and Darby (11) are possibly also explainable by such a hypothesis. An excessive rate of oxidative deamination of amino acids and the concurrent accelerated production of hydrogen peroxide could explain many of the biochemical changes in pernicious anemia and in PGA deficiency in the chick and in the monkey. The high liver weights and enlarged gallbladder of the deficient chicks suggest that an abnormally high rate of destruction of blood elements, or at least of materials normally used in blood production, does occur in PGA deficiency.

An important function of PGA and of liver extract may therefore be the regulation of the activity of flavin-containing enzymes such as xanthine oxidase, L-amino acid oxidase, D-amino acid oxidase, diaphorase, cytochrome reductase, etc. Evidence indicating a depressing effect of PGA on glycine oxidase will be presented shortly. Differences in the action of PGA and of liver extract may result from qualitative and quantitative differences in the ability of each to control adequately all of the enzymes involved.

#### SUMMARY

Weight data are presented on chicks receiving 0, 5, 10, 20, 40, 80, 200, or 1000  $\gamma$  of pteroylglutamic acid (PGA) per 100 gm. of purified diet for a 4 week period.

The ratios of the chick liver weights to body weights were found to be inversely related to the PGA content of the purified diets. The gallbladders of the deficient chicks were greatly enlarged.

The oxygen uptake of liver homogenate from chicks receiving 0, 5, and 10  $\gamma$  of pteroylglutamic acid per 100 gm. of diet was slightly depressed by addition of pteroylglutamic acid. No effect was observed on chick liver tyrosinase activity either by dietary PGA or by PGA added *in vitro*.

The chick liver xanthine oxidase was found to be inversely related to the PGA content of the purified diets. Excess of the vitamin did not reduce the average xanthine oxidase activity to a value as low as that exhibited by chicks on a commercial diet.

The bearing which the findings have on the interpretations of the biochemical changes occurring in pernicious anemia are briefly discussed.

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# CALCIFICATION OF TEETH

## I. COMPOSITION IN RELATION TO BLOOD AND DIET\*

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(Received for publication, July 27, 1948)

These investigations were undertaken to test the hypothesis, of the authors, that there is a relationship between the inorganic composition of teeth and the fluid from which the tooth salts precipitate and that the composition of this fluid in turn is related to the blood serum. If this hypothesis is valid, it should be possible to vary the composition of teeth (during the period of active lime salt deposition), since the composition of blood serum can be regulated by the diet (1-6).

The mineral phase is regarded as essentially the same in teeth and bone (7, 8). The variations in the composition of bone are well known (9) and were shown to be related to that of blood (5, 6). Hitherto no such relationships have been shown for teeth. In fact, some recent investigations indicate that the inorganic composition of enamel is constant (10). There is, however, evidence that teeth are tissues in equilibrium with body fluids. Barnum and Armstrong (11) fed  $P^{32}$  to cats and found that it was present in both enamel and dentin after an interval. They then postulated that dentin phosphate may be in equilibrium with blood phosphate and that enamel phosphate may be in equilibrium with dentin phosphate. Greenberg (12) administered  $Ca^{45}$  to rachitic rats and found that in the absence of vitamin D the uptake of the isotope in the femurs was greater than in the molars but less than in the incisors. In the presence of vitamin D, the uptake was greatest in the femur, less in the incisors, and least in the molars. Vitamin D had a minor influence on teeth but a major influence on bone. Armstrong and Barnum (13) fed  $Ca^{45}$  and  $P^{32}$  to albino rats and measured the amounts found in the teeth. They found that the turnover of the isotopes in the incisor dentin was two-thirds and in the incisor enamel about one-third of that in the femur. The molar dentin was about one-sixth that of the femur and the molar enamel was from 1.5 to 3 per cent of the femur.

These studies with isotopes indicate that the continually growing incisors may undergo changes almost as rapidly as bone and thus were considered suitable for our experiments. It is worth pointing out that the hypothesis

\* Presented in part before the Division of Biological Chemistry at the 112th meeting of the American Chemical Society, New York, September 15-19, 1947.

proposed in the first paragraph applies to teeth only during the period when active calcification takes place. In human teeth active calcification is almost complete before eruption.

The knowledge of the factors that influence the composition of teeth is of theoretical and practical importance, since further studies may reveal some relation between caries and structural, mechanical, and other properties (such as resistance to acids and bacteria) and composition.

In the experiments reported here, evidence is given that there is a relationship between the  $\text{PO}_4:\text{CO}_3$  ratios of enamel, dentin, and blood serum, which in turn is regulated by the diet.

#### EXPERIMENTAL

The three diets used are shown in Table I. The basal diet contained traces of calcium (0.029 per cent) and suboptimal amounts of phosphorus (0.118 per cent).<sup>1</sup> To this basal diet 3.0 per cent  $\text{CaCO}_3$  was added to produce a high calcium-low phosphorus diet (Diet B); 3.0 per cent of anhydrous  $\text{Na}_2\text{HPO}_4$  was added to give a low calcium-high phosphorus diet (Diet C); and 0.4 per cent  $\text{CaCO}_3$  was added to give the low calcium-low phosphorus diet (Diet D). The calcium and phosphorus contents of the diets were determined by replicate analysis. Each diet contained the same amount of either calcium or phosphorus as one of the others.

Young rats, 23 days old, were used. The animals were raised in our laboratory from a Wistar strain stock colony, fed on the standard stock diet of Bills *et al.* (14). Litters were chosen having at least eight animals per litter. Two animals of each litter were sacrificed on the 1st day of the experiment to furnish data on the initial composition of the blood and teeth of the litter. These animals were termed the reference group. It was necessary, owing to the small size of the young animals, to pool the blood and teeth of both animals for analysis. The remaining six animals in each litter were divided into three pairs and each pair placed on one of the three experimental diets. In addition to these diets, one animal of each pair received daily 100 i.u. of vitamin D in 0.1 ml. of corn oil administered orally by means of a pipette. The six groups thus established were caged separately, in a darkened room, to prevent mixing of the diets and to prevent the access of those animals not receiving vitamin D to this factor.

Eleven litters of rats were used. The mean initial weights of the rats were almost identical (see Table II). A reasonable basis for comparing the results of the various groups was established because of the similarity

<sup>1</sup> In these experiments a basal diet, low in phosphate, was desired to obtain greater freedom in changing the Ca:P ratio. This was accomplished by selecting degerminated yellow corn-meal which is low in phosphorus (Hoger Cereal Company, New York).

of heredity, the distribution of litters among the various groups, and the close agreement of the initial weights.

The first group consisting of six litters, and referred to as the short term experiment, was kept on the experimental diets for 30 days, at the end of which time the rats were sacrificed by cutting the jugular vein and the carotid artery. The blood was collected under oil and centrifuged immediately upon clotting. The upper incisors were immediately removed, cleaned from adhering tissues, and prepared for analysis. The second group of animals, consisting of five litters (referred to as the long term experiment), was treated exactly the same as the animals on the short term

TABLE I  
*Composition of Experimental Diets*

Diet	Constituents		Ca	P	Ca:P
		<i>parts</i>	<i>per cent</i>	<i>per cent</i>	<i>mole ratio</i>
B. High calcium-low phosphorus	Basal diet	97			
	Calcium carbonate	3	1.20	0.121	7.67
C. Low calcium-high phosphorus	Basal diet	97			
	Na <sub>2</sub> HPO <sub>4</sub>	3	0.028	0.759	0.029
D. Low calcium-low phosphorus	Basal diet	99.6			
	Calcium carbonate	0.4	0.189	0.124	1.18
Basal	Yellow corn-meal	75			
	Wheat gluten	19			
	Brewers' yeast	5			
	Sodium chloride	1	0.029	0.118	0.191

experiment except that they were kept on the experimental diets for 45 days.

### *Analytical Procedures*

The serum, under oil, was analyzed for CO<sub>2</sub> on 0.1 ml. by the manometric method of Van Slyke and Neill (15), for calcium in duplicate on 0.1 ml. portions by the method of Sobel and Sobel (16), and for inorganic phosphate in duplicate on 0.1 ml. portions by the micro modification of the Fiske-Subbarow method (17, 18), with the micro cups of the Klett-Sumerson photoelectric colorimeter with a 660 mμ filter. Standards were run for CO<sub>2</sub>, calcium, and inorganic phosphate for each set of sera. Standards for CO<sub>2</sub> consisted of a sodium carbonate solution, 1 ml. of which is equivalent to 100 volumes per cent of CO<sub>2</sub>. The standard for calcium consisted of a calcium carbonate solution, 0.1 ml. of which is equivalent to 10.00 γ of calcium. The standard for inorganic phosphate consisted of a solution of KH<sub>2</sub>PO<sub>4</sub>, 5 ml. of which are equivalent to 40.0 γ of P. The precision of the methods is shown in Table III.

The upper incisors were split into fragments and extracted for three overnight periods with two portions of acid- and aldehyde-free ethyl alcohol and one portion of ethyl ether. The fragments were then dried overnight

TABLE II  
*Growth data*

	Dietary group	Initial weight	Final weight	Gain
		gm.	gm.	gm.
Experimental period, 30 days	Reference group	+30.1		
	B	+31.1	+55.0	+23.9
	C	+31.2	+44.5	+13.3
	D	+30.0	+64.9	+34.9
	B + vitamin D	+30.6	+43.6	+13.0
	C + " "	+30.3	+48.5	+18.2
	D + " "	+29.7	+73.5	+43.8
	B - C	-0.1	+11.5	+10.6
	" - D	+1.1	-9.9	-11.0
	C - "	+1.2	-20.4	-21.6
	B + vitamin D - C + vitamin D	+0.3	-4.9	-5.2
	" " " - D + " "	+0.9	-29.9	-30.8
	C + " " - " + " "	+0.6	-25.0	-25.6
	B - (B + vitamin D)	+0.5	+11.4	+10.9
	C - (C + " ")	+0.9	-4.0	-4.9
	D - (D + " ")	+0.3	-8.6	-8.9
Experimental period, 45 days	Reference group	+35.6		
	B	+36.8	+51.9	+15.1
	C	+36.6	+45.9	+9.3
	D	+35.8	+82.1	+46.3
	B + vitamin D	+36.1	+43.4	+7.3
	C + " "	+36.1	+64.5	+28.4
	D + " "	+36.8	+88.4	+51.6
	B - C	+0.2	+6.0	+5.8
	" - D	+1.0	-30.2	-31.2
	C - "	+0.8	-36.2	-37.0
	B + vitamin D - C + vitamin D	0.0	-21.1	-21.1
	" + " " - D + " "	-0.7	-45.0	-44.3
	C - " " - " + " "	-0.7	-23.9	-23.2
	B - (B + vitamin D)	+0.7	+8.5	+7.8
	C - (C + " ")	+0.5	-18.6	-19.1
	D - (D + " ")	-1.0	-6.3	-5.3

at 105°, weighed, and the enamel and dentin separated by the method of Manly and Hodge (19), which was modified to the extent that the tube used for carbonate analysis (20) was employed instead of the centrifuge tube recommended by Manly and Hodge. This was done to obviate the neces-

sity of transferring small amounts of enamel and dentin obtained from the teeth, with the consequent mechanical loss of material. The separated enamel and dentin were dried overnight at 105°, after two acetone washings, and placed in a desiccator till cool and then weighed. These weights were used to calculate the percentages of the inorganic constituents of the enamel and dentin.

The enamel was analyzed for carbonate, calcium, phosphate, and total base as previously described (18), except that the Coleman spectrophotometer was used instead of the visual colorimeter for phosphate. For the carbonate analysis, the gasometric reagents were prepared in 25 per cent sodium chloride for added sensitivity (21). Standard solutions were run for calcium, phosphate, total base, and for carbonate solid mixtures. The

TABLE III

*Precision of the Determinations of Standard Solutions Employed As Controls for Enamel, Dentin, and Blood Serum Analysis*

Mean values (approximately 50 determinations plus-minus average deviations).

Ca*	Ca†	CO <sub>2</sub> ‡	CO <sub>2</sub> §	PO <sub>4</sub>	Total base¶
γ	γ	vol. per cent	wt. per cent	γ	m.eq.
10.03 ± 0.12	199.0 ± 1.7	100.0 ± 1.3	2.00 ± 0.02	39.0 ± 0.5	0.101 ± 0.001

\* 10.00 γ of Ca present in 0.1 ml. used for analysis.

† 200 γ of Ca present in 1.0 ml. of solution used for analysis (bone method).

‡ 100.0 volumes per cent of CO<sub>2</sub> present; 0.1 ml. used for analysis.

§ 2.00 per cent as CO<sub>2</sub> in a mixture of solid NaCl and Na<sub>2</sub>CO<sub>3</sub> (bone method).

|| 40.0 γ of phosphate P present (bone and blood methods).

¶ 0.100 milliequivalent of Ca present in 1.00 ml.

latter consisted of a dry sodium chloride-sodium carbonate mixture containing 2.00 per cent CO<sub>3</sub>. The precision of these determinations is given in Table III.

### Calculations (6, 20)

#### Carbonate

$$\text{Mg. CO}_2 \text{ in sample} = P_{\text{CO}_2} \times \text{factor for CO}_2$$

$$P_{\text{CO}_2} = p_1 - p_2 - c$$

$$c = p_1 - p_2 \text{ for blank analysis}$$

#### Calcium

$$\text{Mg. Ca in sample} = \frac{\text{titer} \times \text{normality of acid} \times 20 \times \text{total volume}}{\text{volume of aliquot used}}$$

#### Phosphate

Klett-Summerson colorimeter:

$$\text{Mg. P in aliquot} = \frac{\text{reading of unknown}}{\text{reading of standard}} \times \text{concentration of standard}$$



For the Coleman spectrophotometer (used for tooth analysis) a calibration curve was employed.

$$\text{Mg. P in sample} = \text{mg. P in aliquot} \times \frac{\text{total volume of solution}}{\text{volume of aliquot used}}$$

### Total Base

$$\text{Milliequivalents total base} = \frac{\text{titer} \times \text{normality of acid} \times \text{total volume of solution}}{\text{volume of aliquot used}}$$

$$\text{mm Ca} = (\text{mg. Ca in enamel or dentin})/40.08$$

$$\text{" PO}_4 = (\text{mg. inorganic P in enamel or dentin})/31.02$$

$$\text{" CO}_3 = (\text{mg. CO}_3 \text{ in enamel or dentin})/60.01$$

$$\text{" \% Ca, PO}_4, \text{CO}_3, \text{ or total base} = (\text{mm Ca, PO}_4, \text{CO}_3, \text{ or total base} \times 100)/\text{mg. weight of dry, fat-free enamel or dentin}$$

$$\text{Ca:PO}_4 \text{ ratio} = \text{mm Ca}:\text{mm PO}_4$$

$$\text{mm residual Ca} = \text{mm total Ca} - \text{mm CO}_3$$

$$\text{Milliequivalents residual} \frac{\text{total base}}{2} = \text{milliequivalents} \frac{\text{total base} - \text{mm CO}_3}{2}$$

$$\text{Residual Ca:PO}_4 = \text{mm residual Ca}:\text{mm PO}_4$$

$$\text{" total base: 2PO}_4 = \text{milliequivalents residual total base: 2 mm PO}_4$$

$$\text{PO}_4:2\text{CO}_3 = \text{mm PO}_4:2 \text{ mm CO}_3$$

The  $\text{PO}_4:2\text{CO}_3 = n$  in the usual way of representing the inorganic composition of bone and teeth  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaCO}_3]_{1.0}$  (7, 8) when the molar ratio of residual  $\text{Ca:PO}_4$  is 1.50. This holds true also when the residual  $\text{Ca:PO}_4$  molar ratio is more than 1.50 if we take the  $\text{CaCO}_3$  as unity. The formula in this case would be  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaCO}_3]_{1.0}[\text{CaX}]_y$ . When the residual  $\text{Ca:PO}_4$  molar ratio is less than 1.50, we have the type of formula represented by  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaCO}_3]_{1.0}[\text{CaHPO}_4]_x$  and  $n$  is then overstated.

The mean results of the enamel and dentin analysis were evaluated by the statistical methods of Fisher (22) as applied to small samples.  $P$  represents the probability that the difference between two means is due to chance. When  $P$  has a value less than 0.05, the difference between two means is statistically significant.

### Weight Changes

The weight changes, including the initial and final weights are given in Table II. Both in the absence and in the presence of vitamin D, Diet D gave the best growth. This is in harmony with the fact that dietary calcium to phosphorus ratios between 1.0 and 2.0 are optimum for best growth (1-3). The absence of vitamin D gave better growth in Group C than in Group D, and in the presence of vitamin D, the reverse was the case. In previous experiments (5) better growth was obtained in Group B than in Group C, since the phosphate level was 0.32 per cent instead of the present 0.12 per cent.

The influence of vitamin D was greatest in Group C. In Group B, there was a reduction which again may be explained on the basis of the relatively high calcium-low phosphorus ratio. On such diets vitamin D diverts the available phosphorus from the soft tissues to the bone. In Group D, in which growth was greatest, the addition of vitamin D had no significant effect. In the absence of vitamin D the rickets produced in Group B were severe and in Group C mild.

TABLE IV  
*Composition of Serum in Relation to Diet (Mean Values)*

	Dietary group	Ca	P	CO <sub>2</sub>	Ca × P	Ca:PO <sub>4</sub>	PO <sub>4</sub> :CO <sub>2</sub>	Dietary Ca:P
		mg. per cent	mg per cent	vol. per cent		molar ratio	molar ratio	molar ratio
Experimental period, 30 days	B	11.7	2.1	60.6	23.1	4.32	0.023	7.67
	C	5.6	7.5	53.7	41.8	0.58	0.101	0.029
	D	9.4	4.7	58.0	44.1	1.55	0.057	1.18
	B + vitamin D	13.3	3.4	62.6	44.9	3.03	0.039	7.67
	C + " "	8.8	8.4	57.6	74.2	0.81	0.108	0.029
	D + " "	11.1	6.0	56.0	65.8	1.43	0.076	1.18
	Reference	11.2	9.5	47.6	105.8	0.92	0.148	
Experimental period, 45 days	B	12.3	1.8	54.1	22.2	5.30	0.024	7.67
	C	5.1	4.9	57.7	25.0	0.81	0.062	0.029
	D	10.0	2.6	50.6	26.0	2.98	0.037	1.18
	B + vitamin D	15.0	3.4	57.6	51.0	3.42	0.043	7.67
	C + " "	8.9	5.2	56.2	46.4	1.33	0.067	0.029
	D + " "	11.2	4.6	55.6	51.5	1.89	0.060	1.18
	Reference	10.9	7.7	53.9	83.8	1.10	0.104	

### *Composition of Blood, Enamel, and Dentin*

*Blood Serum and Diet*—The results obtained from the analysis of the blood sera are presented in Table IV. There is a direct relationship in the order of magnitude of serum Ca:PO<sub>4</sub> and dietary Ca:P ratios. There is an inverse relationship in the order of magnitude of serum PO<sub>4</sub>:CO<sub>2</sub> and dietary Ca:P ratios. Vitamin D raised the Ca × P product in all cases, the per cent rise being more in that member of the calcium-phosphorus pair which was low compared to the normal, but did not completely overcome the influence of the diet. For this reason the differences between the maxima and minima in the Ca:PO<sub>4</sub> and the PO<sub>4</sub>:CO<sub>2</sub> ratios are less, though still in the same order as in the absence of vitamin D. These relationships confirm, even more definitely, the results of previous investigators (1-6).

*Deposition of Inorganic Components As Indicated by Percentages Present*—It is evident from Table V that the density of calcium, carbonate, and total base is decisively higher in the enamel than in the dentin in all cases.

The density of phosphorus is higher in all but two cases. This confirms previous work indicating higher density in enamel (19, 23-26). Dietary calcium, phosphorus, and vitamin D were not decisive in influencing the density of either enamel or dentin, though in the long term experiment the carbonate was highest in Group B and lowest in Group C.

*Enamel, Dentin, and Blood Serum*—The composition of enamel and dentin in relation to blood serum and diet is given in Table VI and the statistical evaluation of the data in Table VII. One is impressed by the fact that the differences that existed in composition were magnified in the long term experiment. The  $\text{PO}_4:\text{CO}_3$  ratios in dentin and enamel arrange them-

TABLE V  
*Composition of Enamel and Dentin (Mean Values)*

The results for total base are given in milliequivalents per cent  $\times 100$ ; for Ca,  $\text{PO}_4$ , and  $\text{CO}_3$  in mm per cent  $\times 100$ .

	Dietary group	Enamel				Dentin			
		Total base	Ca	$\text{PO}_4$	$\text{CO}_3$	Total base	Ca	$\text{PO}_4$	$\text{CO}_3$
Experimental period, 30 days	B	200.0	96.6	50.6	5.31	124.3	61.2	43.6	5.05
	C	199.5	94.3	55.6	5.20	111.3	54.1	39.3	3.67
	D	224.2	108.9	56.0	5.02	118.7	58.5	45.1	4.27
	B + vitamin D	149.0	71.5	40.8	6.57	116.8	57.3	41.5	4.56
	C + " "	166.3	80.3	46.4	4.37	117.5	58.2	44.9	3.83
	D + " "	186.0	87.3	51.7	5.64	119.2	59.0	44.1	4.12
Experimental period, 45 days	Reference	219.0	102.2	47.8	5.99	125.3	59.6	45.2	3.61
	B	181.4	90.1	45.2	6.17	133.8	64.2	44.1	4.15
	C	196.2	96.1	51.5	3.47	124.6	61.5	44.2	2.48
	D	188.6	93.5	46.4	4.00	143.0	68.1	50.4	3.63
	B + vitamin D	188.5	93.2	50.3	6.35	130.8	63.4	46.6	4.46
	C + " "	198.3	98.5	55.1	4.01	132.3	65.4	48.1	3.02
	D + " "	191.6	94.7	47.9	4.32	135.4	67.2	47.2	3.39
	Reference	169.4	84.5	49.8	7.78	124.6	59.9	44.3	3.62

selves in the same order as the serum  $\text{PO}_4:\text{CO}_3$  ratios for the three groups not receiving vitamin A. The same holds true for the three groups receiving vitamin D. When all six groups of the short term experiment are arranged in the order of magnitude of serum  $\text{PO}_4:\text{CO}_3$  ratios, the  $\text{PO}_4:\text{CO}_3$  ratios in both dentin and enamel follow the same order. In the long term experiment the dentin and enamel  $\text{PO}_4:\text{CO}_3$  and serum  $\text{PO}_4:\text{CO}_3$  ratios do not show the same pattern as in the short term experiment when all six groups are arranged in order of magnitude. This may be indirect evidence that vitamin D has an influence. Statistically, the most significant differences are between the two extreme groups; namely, Group B (high calcium-low phosphorus) and Group C (low calcium-high phosphorus).

The  $\text{PO}_4:\text{CO}_2$  ratios of the enamel in all cases are lower than of the corresponding dentin. The difference was most marked in the high calcium-low phosphorus group (Diet B) and least in the low calcium-high phosphorus group (Diet C). In fact the differences in the absence of vitamin D for this group were not statistically significant, though all other differences were statistically significant for corresponding enamels and dentins. In this connection one can point out that the differences in composition between enamel and dentin of humans and dogs (26) and rats (27) may be due to the particular diet given rather than to species differentiation.

TABLE VI

*Composition of Enamel and Dentin in Relation to Blood Serum and Diet (Mean Values)*

The results represent molar ratios.

	Dietary group	Dietary $\text{Ca}:\text{PO}_4$	Serum		Enamel		Dentin	
			$\text{Ca}:\text{PO}_4$	$\text{PO}_4:\text{CO}_2$ *	$\text{Ca}:\text{PO}_4$	$\text{PO}_4:\text{CO}_2$	$\text{Ca}:\text{PO}_4$	$\text{PO}_4:\text{CO}_2$
Experimental period, 30 days	B	7.67	4.32	2.51	1.92	2.08	1.41	4.40
	C	0.029	0.58	10.10	1.68	4.60	1.38	5.43
	D	1.18	1.55	5.86	1.95	2.65	1.31	5.15
	B + vitamin D	7.67	3.03	3.94	1.75	2.43	1.38	4.60
	C + " "	0.029	0.81	10.80	1.74	3.68	1.29	5.98
	D + " "	1.18	1.43	7.75	1.70	3.00	1.36	5.41
	Reference		0.92	14.80	2.20	3.25	1.31	6.56
Experimental period, 45 days	B	7.67	5.30	2.41	1.99	3.71	1.51	5.47
	C	0.029	0.81	6.15	1.85	7.72	1.41	9.31
	D	1.18	2.98	3.72	2.01	5.92	1.36	6.87
	B + vitamin D	7.67	3.42	4.27	1.86	3.62	1.39	5.55
	C + " "	0.029	1.33	6.70	1.77	6.94	1.36	7.97
	D + " "	1.18	1.89	5.99	1.98	5.67	1.41	7.27
	Reference		1.10	10.40	1.68	3.34	1.39	4.88

\*  $\times 100$ .

Not until we understand the influence of diet in a given species can we discuss the differences between enamel and dentin as characteristic of different species.

Though  $\text{Ca}:\text{PO}_4$  ratios of serum undergo wide variations, in the enamel and dentin the ratios undergo no corresponding variations. Statistically there were significant differences in the two extreme groups; namely, the high calcium-low phosphorus and the low calcium-high phosphorus group (Diets B and C). In the short term experiment these differences were so only in the enamel, while in the long term experiment they were true for both enamel and dentin. In our earlier studies on bone (6), significant

[illegible]

TABLE VII—*Concluded*

Line No.	Dietary group	Serum		Enamel		Dentin		Enamel vs. dentin	
		Ca:PO <sub>4</sub>	PO <sub>4</sub> :CO <sub>2</sub>	Ca:PO <sub>4</sub>	PO <sub>4</sub> :2CO <sub>2</sub>	Ca:PO <sub>4</sub>	PO <sub>4</sub> :2CO <sub>2</sub>	Ca:PO <sub>4</sub>	PO <sub>4</sub> :2CO <sub>2</sub>
Experimental period, 45 days									
1'	B vs. C	0.01	0.01	0.01	0.01	0.02	0.01		
2'	" " D	0.05	0.01	0.9	0.01	0.05	0.02		
3'	C " "	0.01	0.01	0.05	0.05	0.8	0.02		
4'	B + vitamin D vs. C + vitamin D	0.05	0.01	0.9	0.01	0.6	0.01		
5'	B + vitamin D vs. D + vitamin D	0.1	0.3	0.2	0.01	0.8	0.1		
6'	C + vitamin D vs. D + vitamin D	0.01	0.6	0.01	0.05	0.4	0.3		
7'	B vs. B + vitamin D	0.2	0.2	0.2	0.8	0.1	0.9		
8'	C " C + " "	0.01	1.00	0.8	0.4	0.7	0.2		
9'	D " D + " "	0.1	0.1	0.7	0.6	0.5	0.1		
10'	Reference vs. B	0.01	0.01	0.02	0.2	0.02	0.3		
11'	" " C	0.01	0.01	0.2	0.01	0.4	0.01		
12'	" " D	0.01	0.01	0.01	0.01	0.6	0.01		
13'	" " B + vitamin D	0.01	0.01	0.1	0.3	1.0	0.01		
14'	" " C + " "	0.01	0.01	0.3	0.01	0.2	0.01		
15'	" " D + " "	0.01	0.01	0.01	0.01	0.8	0.01	0.01	0.01
16'	B							0.01	0.2
17'	C							0.01	0.05
18'	D							0.01	0.05
19'	B + vitamin D							0.01	0.02
20'	C + " "							0.01	0.05
21'	D + " "							0.01	0.01
22'	Reference								

in Table VIII. It is evident that the ratios are still above 1.50 for enamel and below 1.50 for dentin. Thus the differences cannot be accounted for on the basis of the high carbonate content of the enamel.

Another point worth mentioning is that the difference between total base and calcium is not as great as that in bone (6).

Some differences in the two reference groups, shown in Table VI, need to be pointed out. The Ca:PO<sub>4</sub> ratio of the enamel and of the dentin is much higher in the first reference group than in the second. The rats in the two reference groups were raised about 10 months apart and differences in them may be accounted for by the fact that the stock diet used (14) is made up of dried milk, crude casein, yellow corn, alfalfa, cottonseed

meal, brewers' yeast, and salt. Each one of the components, except the salt, can undergo wide variations in composition from batch to batch and thus may cause changes in the fluids that feed the embryo (of which practically nothing is known at the present time) and in the composition of the mother's milk, which is known to undergo change (30).

TABLE VIII  
*Comparison of Residual Total Base to  $PO_4$  and Residual Ca to  $PO_4$  of Enamel and Dentin (Mean Values)*

Dietary group	Enamel						Dentin					
	Residual T.B. — 2	Residual Ca	T.B.:2 $PO_4$	Ca: $PO_4$	Residual T.B.:2 $PO_4$	Residual Ca: $PO_4$	Residual T.B. — 2	Residual Ca	T.B.:2 $PO_4$	Ca: $PO_4$	Residual T.B.:2 $PO_4$	Residual Ca: $PO_4$
Experimental period, 30 days												
B.....	95.7	91.3	1.97	1.92	1.89	1.80	57.1	56.1	1.43	1.41	1.31	1.2
C.....	94.6	89.1	1.79	1.68	1.70	1.60	52.0	50.5	1.42	1.38	1.32	1.2
D.....	107.1	103.9	2.00	1.95	1.92	1.86	55.1	54.2	1.32	1.31	1.22	1.2
B + vitamin D.....	67.9	64.9	1.82	1.75	1.66	1.59	53.8	52.7	1.41	1.38	1.30	1.2
C + " ".....	78.7	75.9	1.79	1.74	1.70	1.63	55.0	54.4	1.31	1.29	1.23	1.2
D + " ".....	87.4	81.7	1.82	1.70	1.69	1.58	55.5	54.9	1.35	1.34	1.26	1.2
Reference.....	103.5	96.2	2.29	2.20	2.17	2.01	59.1	56.0	1.39	1.31	1.31	1.2
Experimental period, 45 days												
B.....	84.5	83.9	2.00	1.99	1.87	1.86	62.7	60.0	1.52	1.51	1.42	1.3
C.....	94.6	92.6	1.90	1.85	1.84	1.80	59.8	59.0	1.41	1.41	1.35	1.3
D.....	90.0	89.5	2.03	2.01	1.94	1.93	67.9	64.5	1.42	1.36	1.35	1.2
B + vitamin D.....	87.9	86.6	1.87	1.86	1.74	1.72	60.9	58.9	1.40	1.39	1.31	1.2
C + " ".....	95.2	94.5	1.80	1.77	1.73	1.71	63.2	62.4	1.38	1.36	1.31	1.3
D + " ".....	91.5	90.4	2.00	1.98	1.91	1.89	64.3	63.8	1.44	1.41	1.36	1.35
Reference.....	76.9	76.7	1.70	1.68	1.54	1.54	58.7	56.6	1.40	1.39	1.33	1.28

T.B. = total base.

#### DISCUSSION

It is evident from these experiments that there is a relationship between the  $PO_4:CO_3$  ratio of blood serum and that of enamel and dentin; this is illustrated in Table IX. For enamel and dentin the  $PO_4:CO_3$  ratio in those groups without vitamin D follows that for serum. The same holds true for those groups given vitamin D. These three groups are arranged in a descending order of dietary Ca:P ratios. Thus the relationship of diet, blood, and teeth is evident. This relationship, in the 30 day experiment,

between blood and teeth is complete. In the 45 day experiment, however, we must segregate the vitamin D-fed groups from the non-vitamin D groups. Vitamin D may manifest an influence that is evident only in the older animals. The effect of vitamin D is not as great on the tooth  $\text{PO}_4:\text{CO}_3$  ratios as one might expect from the changes in the serum  $\text{PO}_4:\text{CO}_3$  ratios. In this connection it is worth mentioning that the density of neither enamel nor dentin was influenced by vitamin D. The rate of turnover of isotopic calcium and phosphorus in the teeth is not markedly influenced by vitamin D (12, 13). This points to an important difference between tooth and bone; namely, that in bone vitamin D exerts a marked influence on the  $\text{PO}_4:\text{CO}_3$  ratio, density of calcification, and rate of turnover of calcium and phosphorus (5, 6, 12, 13). However, it was possible to demon-

TABLE IX

*Relation of Enamel, Dentin  $\text{PO}_4:2\text{CO}_3$ , and Blood Serum  $\text{PO}_4:\text{CO}_3$  (Mean Values)*

Group .....	Reference	B —	D —	C —	B +	D +	C +
Vitamin D .....							
Experimental period, 30 days							
Serum $\times 1000$ .....	148	25.1	58.6	101	39.4	77.5	108
Enamel $\times 100$ .....	325	208	265	460	243	300	368
Dentin $\times 100$ .....	656	440	515	543	460	541	598
Experimental period, 45 days							
Serum $\times 1000$ .....	104	24.1	37.2	61.5	42.7	59.9	67.0
Enamel $\times 100$ .....	334	371	592	772	362	567	694
Dentin $\times 100$ .....	488	547	687	931	555	727	797

strate histological changes in teeth due to lack of vitamin D on a high calcium-low phosphorus diet analogous to that found in bone (31-37).

$\text{PO}_4:\text{CO}_3$  ratios in enamel are in all cases lower than in dentin. The difference was most marked in the high calcium-low phosphorus group (Diet B) and least in the low calcium-high phosphorus group (Diet C). Logan reported (26) that human enamel has a lower carbonate content than human dentin, whereas the carbonate contents of dog enamel and dentin were nearly equal. Bremer (38) found more carbonate in dog dentin than in dog enamel. He also found differences in regions of the enamel and dentin of a single human tooth. Armstrong and Brekhuis (24) reported higher  $\text{PO}_4:\text{CO}_3$  ratios in the enamel than in the dentin of human teeth. In view of the above findings with rats, one can point out that the differences in composition between enamel and dentin may be due to a particular diet rather than to species differentiation. That more than one type



of composition of dentin exists in a given species is indicated by the findings of Bale *et al.* (39). They found that on heating dentin to 900° some samples gave the apatite pattern, while others had the  $\beta$ - $\text{Ca}_3(\text{PO}_4)_2$  lattice. Not until we understand the influence of diet in a given species can we discuss the differences between enamel and dentin as characteristic of various species.

The variations in  $\text{PO}_4:2\text{CO}_2$  ratios in the different groups, which is a measure of  $n$  in the apatite formula,  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaCO}_3]$ , in these teeth, is far greater than those hitherto encountered in bone. In enamel this is from 2.08 to 7.72 and in the dentin from 4.40 to 9.31. In bone the widest spread hitherto found has been 1.86 to 3.33 (6, 9, 40). The question arises as to whether with longer experimental periods (over 45 days) the spread of  $n$  would not be even greater.

The  $\text{Ca}:\text{PO}_4$  ratios<sup>2</sup> of the enamel are distinctly higher than those of the corresponding dentin in all dietary groups (see Tables VI to VIII). Such differences were not found in the reported analyses of human teeth, most of which are given in Armstrong's review (41), nor in dog teeth (26). The ratios of residual total base to  $2\text{PO}_4$  (Table VIII) are all higher than 1.50 for enamel and less than 1.50 for dentin. Our x-ray diffraction studies<sup>3</sup> indicate that apatite is the main lattice in both the enamel and dentin of such teeth. This would imply that in enamel for the apatite formula,  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaX}]$ , X must have some other radical in addition to carbonate, which may be hydroxyl or possibly citrate (28) or fluoride. For dentin, X is some phosphate, probably  $\text{HPO}_4^-$  (6, 29). We hope that x-ray diffraction studies, which are in progress, may throw further light on this subject.

While  $\text{Ca}:\text{PO}_4$  ratios in serum undergo wide variation, no such wide variations are found for  $\text{Ca}:\text{PO}_4$  or residual  $\text{Ca}:\text{PO}_4$  in enamel and dentin. On the whole, the ratios in Group B were higher than those in Group C. In this connection one may note that the variations in  $\text{Ca}:\text{PO}_4$  ratios in bone are relatively slight and were significant only between Groups B and C in the absence of vitamin D (6). It may be concluded that the  $\text{PO}_4:\text{CO}_2$  ratios in bone and teeth are characterized by large variations, whereas the  $\text{Ca}:\text{PO}_4$  ratios are characterized by slight variations.

<sup>2</sup> It must be emphasized that all through these analyses a ground-up bone sample was periodically analyzed with approximately the same amounts of solid and by the same methods as in the case of enamel and dentin. In each instance we obtained the same residual  $\text{Ca}:\text{PO}_4$  ratio, which was between 1.51 and 1.52. The theoretical residual  $\text{Ca}:\text{PO}_4$  ratio is 1.50 for  $[\text{Ca}_3(\text{PO}_4)_2]_n[\text{CaCO}_3]$ . Thus the changes in  $\text{Ca}:\text{PO}_4$  and residual  $\text{Ca}:\text{PO}_4$  ratios must be taken as valid.

<sup>3</sup> Sobel, A. E., Hanok, A., Kirshner, H., and Fankuchen, I., unpublished experiments.

In Table X, data from Experiments 112 and 117 of Logan and Taylor were recalculated (42). It may be noted that for a 33 per cent increase in the  $\text{PO}_4:\text{CO}_3$  ratio of the initial liquid composition the ratio of the solid increased 41 per cent. For a 33 per cent increase in the  $\text{Ca}:\text{PO}_4$  ratio of the initial liquid, the ratio in the solid increased 3 per cent, and the residual  $\text{Ca}:\text{PO}_4$  ratio underwent no change at all. These studies are fragmentary and by no means conclusive; nevertheless they may be taken as clues explaining the relative constancy of  $\text{Ca}:\text{PO}_4$  ratios in teeth and bone and the wide variations in the  $\text{PO}_4:\text{CO}_3$  ratios. A systematic investigation of the inorganic precipitates formed in the presence of various amounts of  $\text{Ca}$ ,  $\text{PO}_4$ , and  $\text{CO}_3$  ions is needed to understand fully the relationships found between the composition of teeth and bone and blood serum.

TABLE X  
 *$\text{PO}_4:2\text{CO}_3$  and  $\text{Ca}:\text{PO}_4$  Ratios of Liquid and Solid (Calculated from Data of Logan and Taylor (42))*

Experiment No., Logan and Taylor (42)	$\text{PO}_4:2\text{CO}_3$		$\text{Ca}:\text{PO}_4$	
	112	117	112	117
	molar ratio	molar ratio	molar ratio	molar ratio
Liquid initially.....	2.39	1.79	1.00	1.33
“ at end of 20 days . . .	1.00	0.17	0.0035	0.0094
Ppt. at end of 20 days.. . .	4.71	3.35	1.54	1.59
			1.44*	1.44*

\* Residual  $\text{Ca}:\text{PO}_4$  ratio.

The relationships established in these studies were found in the continuously growing incisors of rats. The question naturally arises as to whether molars and incisors of species which do not have continuous growth would behave in a similar fashion. The full answer cannot be given at present owing to lack of sufficient evidence. Experiments with isotopes indicate a turnover in even fully erupted molars (11-13). It is our contention that during the period of most active calcification of molars (or of incisors that do not grow continuously) the same laws must essentially hold true. As permanent teeth become more completely calcified, equilibrium with the body fluids slows up to such an extent that only minor changes can be observed. It is our hope that by controlling the diet of a species that has secondary dentition during the period of active calcification of the secondary teeth we will be able to put the above contention to experimental test. An exact quantitative relationship between the composition of blood and teeth cannot be expressed at present, since this would involve a knowledge

showed that a higher degree of reproducibility could be obtained with the latter procedure, provided the flasks were not shaken during the growth period.

*Growth Responses to Cytidine and Uridine*—The growth responses to cytidine and to uridine were determined by adding varying amounts of each in a 1 ml. volume to 25 ml. of basal medium in 125 ml. Erlenmeyer flasks. The flasks were autoclaved, inoculated as previously described (6), and

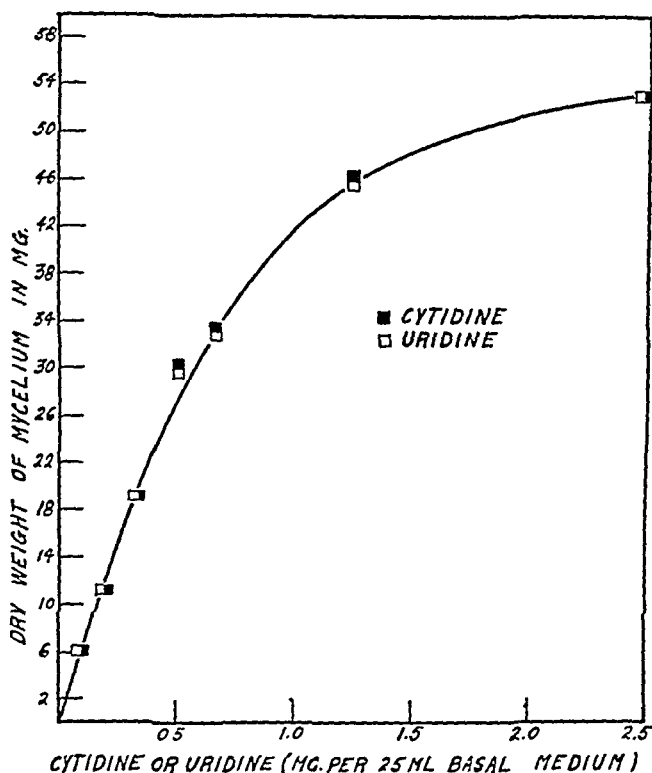


FIG. 1. Growth curve of mutant strain 1298 on uridine (□) and cytidine (■)

placed in an incubator at  $25^{\circ} \pm 0.2^{\circ}$  for 72 hours. The contents were poured into a Petri dish, and the mycelium pad picked up with forceps, squeezed between the fingers, rinsed twice with a small stream of distilled water, and allowed to dry between the folds of a paper towel for 3 to 4 hours at 60–80°. The dry weights of mycelium obtained in this way for different amounts of cytidine and uridine are shown graphically in Fig. 1.

The growth responses to the two compounds are the same within experimental error when the points on the curves are determined in triplicate. Under conditions up to about half maximum growth a relatively high degree

of precision is obtained. The standard deviations in five replicate determinations each at average mycelium weights of 30.7 and 15.5 mg. were 0.6 and 0.4 mg., respectively, corresponding to values for cytidine of  $0.58 \pm 0.02$  mg. and  $0.24 \pm 0.01$  mg. It is evident from Fig. 1 that the greatest relative growth response was found when amounts of supplement giving less than one-half maximum growth were used. As in other *Neurospora* assays the growth curve is approximately linear up to a dry weight of about 20 mg. The relation between weight of mycelium and cytidine or uridine over this range is given with an accuracy of from 5 to 10 per cent by the equation, cytidine or uridine in mg. =  $0.0165 \times$  weight of mycelium in mg.

The volume of solution containing the growth supplement may be increased up to about 5 ml. per 25 ml. of basal medium without significantly affecting the amount of growth. Further dilution, however, probably causes slightly less growth. Of greater importance is the age of the culture used for inoculation. Curves similar to that shown were obtained provided the culture was less than about 5 weeks old. Those older than 5 weeks gave slightly less growth at the different concentrations of supplement.

A procedure similar to that used for obtaining the standard curve is employed for the assay of an unknown solution. Since growth of the mold is inhibited by solutions that are too acid or too alkaline, it is necessary to adjust the pH of the basal medium containing the unknown solution before autoclaving. A convenient method for doing this is to use bromocresol green as an inside indicator and to adjust the pH to 4.5 with acid or alkali as needed (7). Sodium chloride or sodium sulfate in amounts up to about 100 mg. per 25 ml. of basal medium are not inhibitory. The mold, however, is highly sensitive to traces of phosphotungstic acid used for the fractionation of the purine and pyrimidine components or to traces of heavy metals like lead or silver. It is also inhibited by traces of detergents, and glassware washed by a detergent solution should be acid-rinsed before using. It is usually possible to tell whether or not a partial inhibition is present from the type of mold growth obtained. While a single, small mycelium mat is present at lower concentrations of growth supplement in the absence of inhibition, many small foci of growth are sometimes found if an inhibitory substance is present. In other cases partial inhibition is indicated by the slimy nature of the mycelium pad. When inhibition is suspected, it is advisable to carry out additional assays with smaller and larger amounts of the unknown solution. If an inhibitory substance is present, the larger volume of the unknown solution usually results in a relatively greater degree of inhibition, while the opposite is true if less of the unknown is used. It is desirable, therefore, to show that the same value for cytidine or uridine is obtained at different levels of growth.

*Cytidine Phosphotungstate*—As cytidine and uridine occur together in ribonucleic acid, the use of mutant 1298 for the assay of either compound depends on the success with which they can be separated from each other. As cytidine possesses a basic amino group, a number of acids which form relatively insoluble salts with organic bases were investigated to determine their usefulness as possible precipitating agents. Of the five acids studied, picric, picrolonic, nitranilic, flavianic, and phospho-12-tungstic (8), the last gave the most insoluble salt. When sufficient phosphotungstic acid was added to a warm cytidine solution in 1 N hydrochloric acid to give a 2 to 3 mg. per ml. excess, beautifully crystalline prismatic needles of cytidine phosphotungstate separated as shown in Fig. 2. A sample recrystallized once from 1 N hydrochloric acid and air-dried gave the following analyses:<sup>1</sup> H<sub>2</sub>O 8.92, H<sub>3</sub>PO<sub>4</sub>·12WO<sub>3</sub> 78.7, C 5.41, H 1.64 per cent. These compare

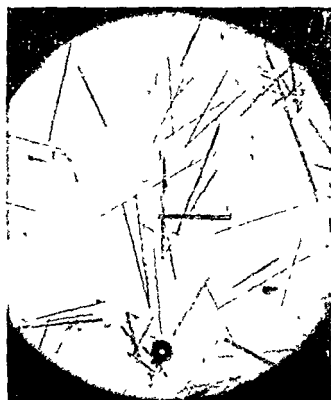


FIG. 2. Cytidine phosphotungstate (magnification about 100 ×)

favorably with theoretical values of H<sub>2</sub>O 8.78, H<sub>3</sub>PO<sub>4</sub>·12WO<sub>3</sub> 78.2, C 5.85, and H 1.72 per cent for (C<sub>9</sub>H<sub>12</sub>O<sub>5</sub>N<sub>3</sub>)<sub>2</sub>·H<sub>3</sub>PO<sub>4</sub>·12WO<sub>3</sub>·18H<sub>2</sub>O. Under similar conditions uridine gave no precipitate with phosphotungstic acid.

*Solubility of Cytidine Phosphotungstate*—The solubility of cytidine phosphotungstate in 1 N hydrochloric acid at 0° was determined from the supersaturated side by precipitating cytidine in the presence of a 2 to 3 mg. per ml. excess of phosphotungstic acid. The filtrate was treated with excess ammonium chloride in 1 N hydrochloric acid to remove the excess phosphotungstic acid,<sup>2</sup> and the filtrate was assayed for cytidine with mutant

<sup>1</sup> C and H analyses by Laboratory of Microchemistry, 366 Fifth Avenue, New York.

<sup>2</sup> The solubility of ammonium phosphotungstate in 1 N hydrochloric acid is less than 0.002 mg. of nitrogen per ml. as shown by the fact that such a concentration of ammonium sulfate nitrogen gives a precipitate with phosphotungstic acid under these conditions.

1298. An average value of 0.08 mg. of cytidine per ml. of the original cytidine phosphotungstate filtrate was found, indicating a nearly quantitative precipitation of cytidine under these conditions.

*Fractionation of Cytidine and Uridine*—The following experiment illustrates the degree of success with which cytidine was separated from an equal amount of uridine. To 20 ml. of warm 1 N hydrochloric acid containing 10 mg. each of cytidine and uridine, 80 mg. of phosphotungstic acid dissolved in 5 ml. of 1 N acid were added. A crystalline precipitate of cytidine phosphotungstate separated. The suspension was allowed to stand in ice in the cold room overnight and was filtered in the cold by gravity. To 20 ml. of this filtrate 75 mg. of ammonium chloride dissolved in 0.5 ml. of 1 N acid were added. After standing overnight in the cold room, the suspension containing ammonium phosphotungstate was filtered and 1 ml. aliquots of the filtrate were assayed for activity with the mold. An average dry weight of mycelium corresponding to 0.51 mg. of uridine was found as compared to the calculated value of 0.47 mg. if the value for the solubility of cytidine phosphotungstate given above, is used and it is assumed that no uridine was precipitated. Other experiments gave similar results indicating that the two compounds could be fractionated by this procedure from relatively dilute solutions with a high degree of success.

*Hydrolysis and Assay of Ribonucleic Acid*—The original procedure attempted for the hydrolysis of ribonucleic acid to ribonucleosides was that given by Jones (p. 112 (9)) for the preparation of ribonucleosides. Assay of a hydrolysate obtained by this method from a sample of purified ammonium nucleate (4) gave a value of 15.2 per cent total pyrimidine nucleosides as compared to that of 34 per cent expected for a statistical tetranucleotide composed of equimolar quantities of adenylic, guanylic, cytidylic, and uridylic acids and containing 10 per cent water, the approximate amount found in most samples of yeast nucleic acid. The surprisingly low value suggested an incomplete hydrolysis and further experiments were carried out to determine the maximum yield of pyrimidine nucleosides which could be obtained after heating for various lengths of time up to 8 hours. Analyses for inorganic and total phosphate were made as well after the 4 and 8 hour periods.

Two concentrations of ammonium nucleate in 2.5 per cent ammonia, namely 20 per cent, the concentration used by Jones, and 2.25 per cent, were employed in the hydrolysis experiments. In the first case 100 mg. were employed in the hydrolysis experiments. In the first case 100 mg. samples in 0.5 ml. and in the second 22.5 mg. samples in 1 ml. in small sealed Pyrex tubes were heated in an oil bath at 140–150°. In the first experiment tubes were taken from the bath at the end of 1, 2, 3, hours and in the second at the end of 4 and 8 hours, and their contents removed, diluted with distilled water, and assayed for activity. Average values expressed as per cent total pyrimidine nucleoside

molar ratios of pyrimidine nucleoside to total phosphate are shown in Table I in comparison with the ratio expected from a statistical tetranucleotide. It may be seen that values corresponding to about 20 per cent pyrimidine nucleosides and a ratio of 0.36 moles of pyrimidine nucleoside per mole of phosphorus were obtained with the more concentrated as well as the less concentrated nucleic acid hydrolysates after 3, 4, and 8 hours. In comparison with the value calculated from the tetranucleotide theory, the largest value found corresponds to about 78 per cent of that expected. The difference between the 4 and the 8 hour values in the less concentrated sample, 0.39 as compared to 0.36, is not highly significant in terms of the precision of the assay procedure, but the results indicate, in agreement with

TABLE I

*Total Pyrimidine Nucleosides and Inorganic Phosphate Found after Heating Yeast Ribonucleic Acid in 2.5 Per Cent Ammonia at 140-150° for Varying Lengths of Time*

Experiment No.	Length of time heated	Total pyrimidine nucleosides	Inorganic phosphate	<u>Pyrimidine nucleoside found</u> <u>Total phosphate</u>
	hrs.	per cent	per cent	mole per mole P
1	1	2.4		0.043
	2	15.1		0.27
	3	20.0		0.36
	4	21.6		0.38
2	4	21.7	6.72	0.39
	8	20.0	7.3	0.36
Statistical tetranucleotide . .				0.50

In Experiment 1 the nucleic acid concentration was 20 per cent and in Experiment 2, 2.25 per cent. Phosphorus content of ammonium nucleate used = 7.14 per cent

other experiments on the stability of the pyrimidine nucleosides in weak alkali, that these compounds are destroyed to a slight extent during the longer period of heating. If it is assumed that a similar loss took place during the first 4 hours of hydrolysis, the highest value for the ratio of pyrimidine nucleosides to phosphorus becomes 0.42 in comparison with the theoretical of 0.50. Inorganic phosphate after 4 and 8 hours corresponded to 94 and 102 per cent respectively of the total phosphorus, showing that the nucleic acid was not completely hydrolyzed to the nucleoside stage under the conditions used until after 4 hours.

As adenosine is present in the hydrolysates mentioned above and is known to inhibit the utilization of cytidine and uridine by this strain of *Neurospora* if present in sufficient concentration (10), the low values for total pyrimidine nucleosides might be explained on this basis. An

examination of published data (4) for the ratios of guanine and adenine to phosphorus in the same nucleic acid preparation, however, revealed that the ratio of adenosine found previously to the pyrimidine nucleoside content given in this paper was not sufficiently high to result in inhibition provided that this sample of nucleic acid contained approximately equal quantities of both cytidine and uridine.

A few experiments were performed in which aliquots of hydrolysates prepared after 4 and 8 hours of heating were fractionated by phosphotungstic acid to separate the purine components and cytidine from the uridine present. Assays of the resulting filtrates gave values for uridine very nearly one-half those found for both cytidine and uridine before fractionation. These results are therefore in agreement with the older data (2) that cytidine and uridine are present in equimolar quantities in yeast ribonucleic acid. A number of fractionation experiments on larger samples and on other yeast nucleic acid preparations which will be published in a subsequent paper<sup>3</sup> led to the same conclusion.

#### SUMMARY

A method is described for the determination of cytidine and uridine, by means of the pyrimidine-deficient mutant strain of *Neurospora*, No. 1298. The method permits the determination of these pyrimidine ribonucleosides over the concentration range from about 0.05 mg. to about 0.6 mg. in a volume of 5 ml. or less with a standard deviation of about 5 per cent. Cytidine forms a relatively insoluble, crystalline salt with phospho-12-tungstic acid,  $\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3 \cdot 7\text{H}_2\text{O}$ , having a solubility in 1 N hydrochloric acid of 0.08 mg. per ml. (as cytidine) and can be separated from uridine in known mixtures with a high degree of success. The assay of hydrolysates prepared by heating samples of purified ammonium nucleate from yeast with 2.5 per cent ammonia at 140–150° for varying periods of time shows that the concentration of pyrimidine nucleosides reaches a maximum after 4 hours. The amount found for this preparation was 23.4 per cent or, expressed in relation to its phosphorus content, 0.42 mole of pyrimidine nucleoside per mole of phosphorus. Fractionation experiments with phosphotungstic acid showed that equal amounts of cytidine and uridine were present.

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# PURINE AND PYRIMIDINE ANTAGONISM IN A PYRIMIDINE-DEFICIENT MUTANT OF *NEUROSPORA*

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(Received for publication, June 1, 1948)

Inhibitions of the growth of organisms by closely related structural analogues of several water-soluble vitamins are well known. Similar types of antagonism in the case of purine and pyrimidine metabolites are those between the purine bases, adenine and guanine, and benzimidazole (1) and between barbituric acid and uracil in the growth of *Staphylococcus aureus* (2). In such cases the antagonism is usually considered to be due to competition between the analogue and the metabolite in enzyme systems where the normal metabolite is involved.

Antagonisms between two structurally related, naturally occurring substances are less common but have been demonstrated to exist between amino acids of similar structure (3, 4) and between thiamine and pyridoxine (5). A most striking relationship between normal metabolites is that reported by Raska (6) in which pellagra was produced experimentally in dogs when adenine alone or in conjunction with phosphate was fed in daily doses of 400 to 500 mg. An explanation similar to that mentioned above for the antagonism between metabolites and structural analogues not occurring naturally has also been offered for the closely related natural substances.

The present study is concerned with the inhibition of growth of the pyrimidine-deficient *Neurospora* mutant, No. 1298, by the naturally occurring purine ribonucleotides and ribonucleosides. This experimentally produced strain, unlike the wild type, is unable to synthesize the pyrimidine ribonucleosides on a medium containing inorganic salts, carbohydrate, and biotin. Normal growth takes place, however, when the medium is supplemented with either cytidine or uridine or the corresponding nucleotides (7). It has been found that adenosine and adenosine-3-phosphate (yeast adenylic acid) inhibit the utilization of the pyrimidine compounds to a varying degree. An amount of adenine nucleoside which is sufficient to inhibit growth completely on the quantity of cytidine used has no inhibitory effect on an equivalent amount of uridine. The addition of an equimolar amount of uridine to a mixture of cytidine and adenosine in

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which no growth takes place results in the elimination of the antagonism. In contrast to the effect of adenosine and adenylic acid on this mutant strain of *Neurospora*, adenine shows no inhibitory properties at comparable concentrations. A similar inhibitory effect on the utilization of the pyrimidine nucleosides was found for guanosine and guanylic acid, but larger amounts of these compounds were required to produce inhibition under the same conditions. Guanine like adenine failed to cause inhibition at moderate concentrations.

#### EXPERIMENTAL

The growth response of the mold to various concentrations of supplements and inhibitors as compared to that in the absence of inhibitor was determined from the dry weight of mycelium produced in liquid culture after incubation for 3 days at 25°. The composition of the basal medium, the method of inoculation, and the determination of the weight of mycelium were the same as previously described (7, 8). The concentrations of the pyrimidine derivatives used in the determination of the inhibitory effects of the purine compounds were those which produced an approximately half maximum growth of the mold. In this range an amount of mold which can be readily weighed is obtained, and growth response is most sensitive to small changes in the concentration of added supplement. The growth of the mold was found from the average value of determinations made in triplicate.

Uridylic acid, in the form of the diammonium salt, cytidylic acid, uridine,<sup>1</sup> and guanine<sup>2</sup> were prepared by methods devised in this laboratory (9). Guanosine was isolated from yeast nucleic acid as described by Levene (10). The cytidine, guanylic acid, adenosine, and adenosine-3-phosphate were commercial samples.<sup>3</sup>

*Antagonism by Adenosine and Adenosine-3-phosphate*—The effect of adenine, adenosine, and adenosine-3-phosphate on the growth activity of cytidine, uridine, cytidylic acid, and uridylic acid was determined by adding increasing amounts of each purine compound to the basal medium supplemented with a constant amount of growth factor. The amount of cytidine or uridine used was 0.5 mg. per 25 ml. of basal medium. The growth of the mold in the presence of varying amounts of adenine, adenosine, and adenosine-3-phosphate was determined and plotted as the percentage of growth obtained in the absence of inhibition. The data for

<sup>1</sup> Loring, H. S., and Ploeser, J. McT., unpublished work.

<sup>2</sup> Loring, H. S., and Ali, S. A., unpublished work.

<sup>3</sup> Cytidine and guanylic acid were kindly provided by the National Biochemical Corporation, New York. Adenosine and adenosine-3-phosphate were obtained from Schwarz Laboratories, Inc., New York.

cytidine and the three purine compounds and for uridine and adenosine are shown in Fig. 1. It can be seen that adenosine was twice as inhibitory of cytidine activity as was adenosine-3-phosphate. When uridine was used as the growth factor, approximately five times as much adenosine was required to produce the same degree of inhibition. Free adenine failed to inhibit the growth of the mold on cytidine at a concentration equivalent to 0.6 mg. of adenosine and indeed was slightly stimulatory at some concentrations. Similarly, no significant inhibition of uridine in the presence of adenine was observed at a concentration equivalent to 4.0 mg. of adenosine.

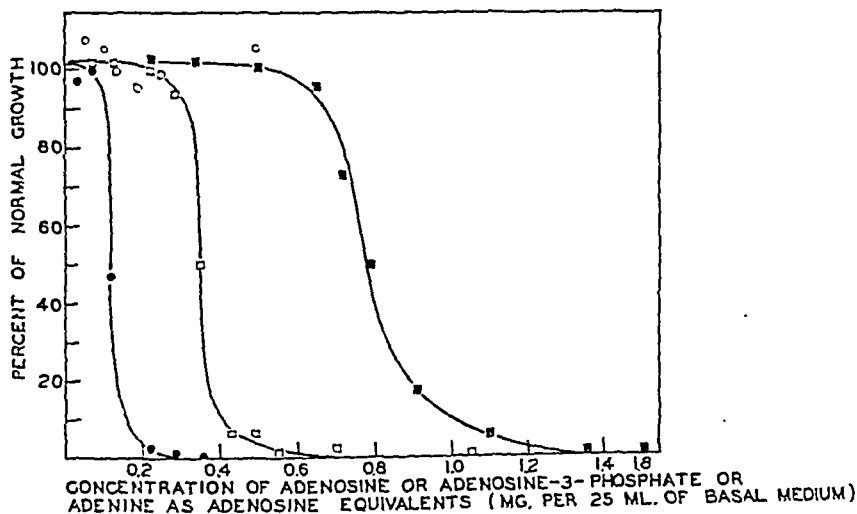


FIG. 1. The effect of adenine, adenosine, and adenosine-3-phosphate expressed as adenosine or adenosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ adenine, ● adenosine, and □ adenosine-3-phosphate in the presence of 0.5 mg. of cytidine; ■ adenosine in the presence of 0.5 mg. of uridine.

In the case of cytidylic acid and uridylic acid, the amounts employed to give about half maximum growth were 1 mg. of cytidylic acid and 1 mg. of diammonium uridyate per 25 ml. of basal medium. Addition of the three adenine compounds in similar amounts to those used for the pyrimidine nucleosides gave inhibition curves of the same type as those shown in Fig. 1. Free adenine as with cytidine and uridine did not affect the utilization of the pyrimidine ribonucleotides. The molar ratios of antagonist to metabolite to give 50 per cent inhibition in the case of the four pyrimidine compounds and adenosine and adenosine-3-phosphate were calculated from the respective inhibition curves and are given in Table I. Of the four pyrimidine compounds it may be seen that cytidylic acid was the most readily inhibited, the molar ratio of adenosine to cytidylic acid for 50 per

cent inhibition being 0.13. In contrast to cytidylic acid, the inhibition ratio for adenosine and uridylic acid was 0.41. It is evident that adenosine-3-phosphate was less inhibitory in all cases than the corresponding nucleoside. Cytidine, like cytidylic acid, was more strongly inhibited than uridine, but each pyrimidine nucleoside was affected to a lesser degree by adenosine than was the corresponding nucleotide.

*Antagonism by Guanosine and Guanosine-3-phosphate*—An entirely analogous situation was found in the case of the guanine compounds. Guanosine and guanylic acid inhibited the utilization of each of the four pyrimidine nucleosides or nucleotides while guanine at comparable concentrations had no effect. The effect of guanosine and guanosine-3-

TABLE I  
*Molar Ratios of Antagonist to Metabolite for 50 Per Cent Inhibition*

Metabolite	Weight metabolite used per 25 ml. medium	Antagonist	Moles antagonist / Moles metabolite
	mg.		
Cytidylic acid	1	Adenylic acid	0.27
“ “	1	Adenosine	0.13
Cytidine	0.5	Adenylic acid	0.60
“	0.5	Adenosine	0.24
Uridylic acid	0.86	Adenylic acid	0.6
“ “	0.86	Adenosine	0.41
Uridine	0.5	Adenylic acid	3.2
“	0.5	Adenosine	1.4
Cytidine	0.5	Guanosine	0.68
“	0.5	Guanylic acid	1.29
Uridine	0.5	Guanosine	3.0
“	0.5	Guanylic acid	7.8

phosphate on mold growth in the presence of 0.5 mg. of cytidine or uridine is shown in Fig. 2. It may be seen that approximately twice as much guanosine or guanylic acid was required to produce the same amount of inhibition as for the adenine compounds. Guanine in an amount equivalent to 10 mg. of guanosine per 25 ml. of basal medium did not affect the growth of the mold in the presence of 0.5 mg. of cytidine or uridine. The molar ratios of guanosine or guanylic acid to cytidine and uridine to produce 50 per cent inhibition are also shown in Table I.

*Antagonism in Mixtures of Pyrimidine Nucleosides and Nucleotides*—The surprising difference in the ability of adenosine to inhibit growth on uridine as compared to cytidine suggested that the antagonism was involved to a different degree in the reactions concerned in the utilization of the two compounds. If the reaction inhibited was the deamination of cytidine to

uridine rather than the utilization of cytidine *per se*, then it should be possible to eliminate the inhibition of cytidine by the addition of sufficient uridine to avoid the necessity of deamination. It was desirable, therefore, to determine the amount of uridine that would cause the reversal of the antagonism in an inhibitory mixture of cytidine and adenosine. A series of flasks containing 0.25 mg. of cytidine and 0.27 mg. of adenosine in 25 ml. of basal medium was supplemented with increasing amounts of uridine from 0.05 to 0.5 mg. The molar ratio of adenosine to cytidine was 1.0, which in the absence of uridine produces complete inhibition. The effect of the

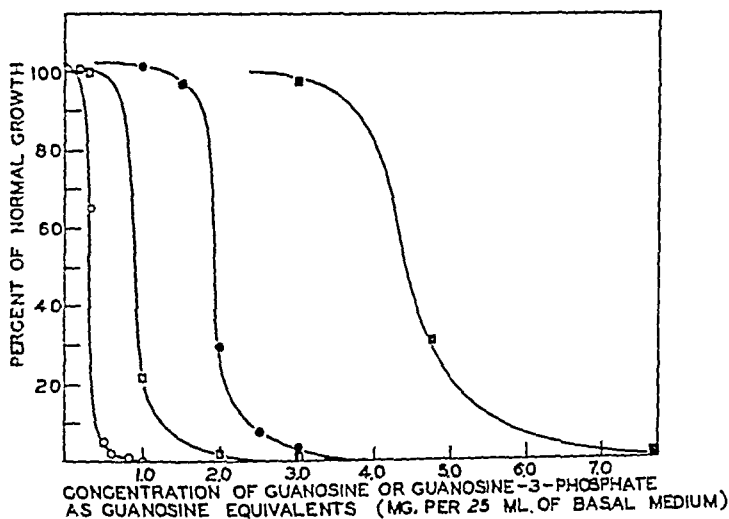


FIG. 2. The effect of guanosine or guanylic acid expressed as guanosine or guanosine equivalents on the growth of *Neurospora* mutant No. 1298; ○ guanosine, and □ guanylic acid in the presence of 0.5 mg. of cytidine; ● guanosine, and ■ guanylic acid in the presence of 0.5 mg. of uridine.

addition of the uridine is shown in Fig. 3, A, where the weight of mycelium found for each concentration of uridine was plotted against the total weight in mg. of cytidine and uridine used as the growth supplement. The curve showing the growth of the mold on either pyrimidine nucleoside in the absence of inhibitor is also presented. It may be seen that the growth-promoting properties of the mixtures were almost completely inhibited until the molar ratio of the cytidine to uridine approached 1. When the ratio reached 1, the inhibition was strikingly eliminated, and as more uridine was added, the amount of growth was approximately that found with either cytidine or uridine when no inhibitor was present. As shown in the cytidine-adenosine curve in Fig. 1, 0.27 mg. of adenosine in the

presence of 0.5 mg. of cytidine, a molar ratio of 0.5, gave 98 per cent inhibition. Thus it is evident that the inhibition was a specific one and that the addition of an equivalent quantity of cytidine instead of uridine would not have overcome the effect of the adenosine.

An experiment performed with 0.4 mg. of cytidylic acid and 0.18 mg. of adenosine, an amount giving complete inhibition, gave a similar elimination of antagonism when increasing amounts of uridylic acid were added. Inhibition was nearly 100 per cent when the ratio of uridylic acid to cytidylic acid was less than 1, but, when the ratio became 1, inhibition no longer was observed. In this case when an additional equivalent of cytidylic acid

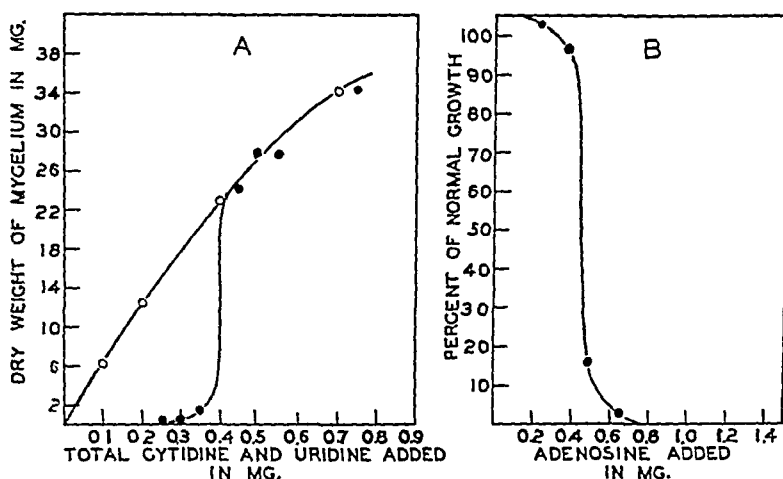


FIG. 3, A. The effect of uridine on an inhibitory mixture of 0.25 mg. of cytidine and 0.27 mg. of adenosine in comparison with the normal growth curve on cytidine or uridine; O normal growth curve on cytidine or uridine, ● growth curve on cytidine-adenosine mixture with varying amounts of uridine.

FIG. 3, B. The effect of adenosine on an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine.

was added to the 0.4 mg. of cytidylic acid-0.18 mg. of adenosine mixture, the amount of growth corresponded to about 95 per cent inhibition.

In an attempt to reverse the guanosine inhibition of cytidine, increasing amounts of uridine were added to a mixture of cytidine and guanosine which is completely inhibitory (0.25 mg. of cytidine and 0.5 mg. of guanosine; cf. Fig. 2). The antagonism was also eliminated in this case by the addition of an equimolar amount of uridine, 0.25 mg. of the latter permitting growth equal to 82 per cent of that expected from either 0.5 mg. of cytidine or the same quantity of uridine with no inhibitor added.

The striking elimination of the inhibition of cytidine by an equimolar amount of uridine provided evidence that the antagonism of adenosine was

concerned in a highly specific way with the deamination of cytidine to uridine, possibly by inhibition of a cytidine deaminase present in the mold. A possible explanation of the antagonism for uridine alone could be the blocking of the reverse reaction, the amination of uridine to cytidine. A larger amount of adenosine would be required for the blocking of this reaction than for the deamination, as shown by the larger amounts required to inhibit growth on uridine. If this were the case, one would expect the amount of adenosine which inhibits the utilization of uridine alone to have no effect on an equimolar mixture of cytidine and uridine, both being available for growth.

To study this question an equimolar mixture of 0.25 mg. of cytidine and 0.25 mg. of uridine was supplemented with different amounts of adenosine from 0.23 to 1.4 mg., and the amount of mold growth determined in each case. The mycelium weights expressed as per cent of growth in the absence of inhibitor and the amounts of adenosine added are shown graphically in Fig. 3, *B*. When 0.27 mg. of adenosine was used, an equimolar mixture of all three components was present, and the growth of the mold was not inhibited, as was expected from the curve shown in Fig. 3, *A*. However, as the ratio of adenosine to cytidine and uridine was increased, mold growth was inhibited in a fashion similar to that found for uridine alone. Thus for 50 per cent inhibition, the same adenosine-uridine ratio of about 1.4 was found in this experiment where cytidine was present as with uridine and adenosine alone. The effect of adenosine on uridine is probably concerned, therefore, with the utilization of uridine for growth directly rather than with its conversion to cytidine.

To ascertain whether the inhibitory effects of adenosine and guanosine on a mixture of cytidine and uridine were additive, increasing amounts of guanosine from 0.2 mg. to 1.0 mg. were added to flasks containing 0.25 mg. of cytidine, 0.25 mg. of uridine, and 0.45 mg. of adenosine in 25 ml. of basal medium. This mixture allows about 30 per cent of the normal growth of the mold to take place; cf. Fig. 3, *B*. The addition of the guanosine resulted in further inhibition.

To demonstrate that the antagonism observed between adenosine and cytidine is a competitive one, *i.e.*, that a constant ratio of antagonist to metabolite will give the same degree of growth regardless of the actual concentration of metabolite present, flasks were set up containing 0.25, 0.50, 0.75, and 1.0 mg. of cytidine per 25 ml. of basal medium. To these were added 0.07, 0.14, 0.21, and 0.28 mg. of adenosine, respectively. The percentage of normal growth obtained (as calculated from the standard growth curve (8)) was approximately the same at each concentration level of antagonist and metabolite, *i.e.*, 8, 6, 9, and 7 per cent, respectively.

Although the antagonism between cytidine and adenosine was also



demonstrated to exist in the pyrimidine-deficient mutant of *Neurospora* No. 263-1895-3a<sup>4</sup> (7), it could not be shown in the wild type which is able to synthesize its pyrimidine requirements. In determining this fact 5 mg. of adenosine were added to 25 ml. of basal medium, and the growth of the wild type measured in the usual way. The amount of growth did not differ significantly in the presence of adenosine from that found with the unsupplemented medium alone.

#### DISCUSSION

The *Neurospora* mutant No. 1298, unlike its wild type counterpart, fails to grow on the basal medium alone but grows when the medium is supplemented with either of the two pyrimidine ribonucleosides or ribonucleotides. As these compounds are essential constituents of ribonucleic acid, it is evident that the failure of growth in their absence is due to a deficiency in ribonucleic acid synthesis. Because normal growth is obtained with either cytidine or uridine or the corresponding nucleotides, it is also apparent that the mold can accomplish the amination of uridine with the formation of cytidine or the deamination of cytidine to uridine as well as the phosphorylations necessary for the formation of nucleic acid from the nucleosides.

The difference in the levels at which cytidine and uridine are inhibited suggests that the inhibition of at least two different reactions is involved. Evidence that the deamination of cytidine to uridine is inhibited is provided by the fact that cytidine was more readily inhibited than uridine and because the cytidine inhibition could be readily eliminated by the presence of an equimolar quantity of uridine. In the latter instance the conversion of cytidine to uridine was no longer required for nucleic acid synthesis and the inhibition of this reaction would not be expected to have the same inhibitory effect on the utilization of the two compounds for growth. At concentrations of adenosine which inhibited growth on uridine, however, it appears that it is the utilization of uridine which is affected rather than its conversion to cytidine, as this inhibition was not removed by the presence of cytidine. The nature of the reaction concerned in this case is not apparent.

The fact that the pyrimidine nucleotides, cytidylic and uridylic acids, are more strongly inhibited by adenosine than are the corresponding nucleosides is in agreement with their less efficient utilization for growth. Similarly adenosine-3-phosphate was less inhibitory than adenosine. These results are in agreement with several others which indicate that the

<sup>4</sup> This mutant was kindly provided by Dr. H. K. Mitchell, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

nucleosides may play a more central rôle in nucleic acid metabolism than either the free bases or their nucleotides.

The absence of adenosine inhibition in the wild type organism is in keeping with other observations that no inhibition is produced by closely related structural analogues when the substance concerned is not required for growth (11). In the wild type *Neurospora* an efficient mechanism may be present for the conversion of adenosine to adenine which later was found in these experiments not to be inhibitory. An alternative explanation may be that pyrimidine synthesis can be stimulated to balance the increased amount of adenosine present.

Of interest is the highly specific nature of the antagonism of the pyrimidine nucleosides by adenosine and guanosine and the striking reversal of the adenosine-cytidine inhibition by uridine. Unlike most inhibitions by closely related structural analogues in which high antagonist-metabolite ratios are necessary to produce inhibition, growth on cytidine was completely inhibited by an equimolar amount of adenosine. Similarly such a completely inhibitory mixture in the presence of a molecular equivalent of uridine behaved as if no antagonist at all were present. These experiments demonstrate the pronounced effect of the purine and pyrimidine nucleosides on growth in this strain of *Neurospora* and suggest a similar function in the control of growth in other organisms.

#### SUMMARY

The utilization of the pyrimidine ribonucleosides and ribonucleotides for growth by the pyrimidine-deficient mutant of *Neurospora* No. 1298 can be completely inhibited by the addition of adenosine or adenosine-3-phosphate to the culture medium. Adenosine is the most active antagonist, adenosine-3-phosphate is somewhat less so, and adenine has no antagonistic effect when added in comparable concentrations. The nucleotides are more readily inhibited than the nucleosides, and cytidylic acid and cytidine require less adenosine or adenosine-3-phosphate for inhibition than do uridylic acid or uridine. Guanosine and guanylic acid also inhibit the utilization of the pyrimidine compounds, but somewhat larger amounts are required. Guanine like adenine shows no inhibitory action at moderate concentrations.

The inhibition of cytidine by adenosine is strikingly reversed by the addition of an amount of uridine equal to the cytidine present. Uridine, however, is inhibited by the same concentration of adenosine regardless of whether or not an equimolar quantity of cytidine is present. These results suggest that at least two reactions may be involved in the inhibition, namely, the deamination of cytidine to uridine and the utilization of uridine itself for the synthesis of ribonucleic acid by the mold.

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## GROWTH AND LIPOTROPISM

### II. THE EFFECTS OF DIETARY METHIONINE, CYSTINE, AND CHOLINE IN THE YOUNG WHITE RAT

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(Received for publication, June 12, 1948)

In a previous study (1) it was shown that the total methionine requirement of rats of approximately 170 gm. initial body weight receiving a choline-free basal diet was 1200 mg. per 100 gm. of diet. This total was differentiated into a growth requirement of 600 mg. and a lipotropic requirement of 600 mg. The basal diet contained 100 mg. of cystine per 100 gm.; additional cystine did not exhibit any effect on growth or any antilipotropic activity. When the basal diet was supplemented with 100 mg. of choline per 100 gm., there was a maximum lowering of liver fat with no increase in the growth rate. Womack and Rose (2), using different experimental conditions, reported that 500 mg. of methionine and 100 mg. of cystine per 100 gm. of diet were adequate to support optimum growth in rats of an initial body weight of approximately 50 gm. Rats of this age are entering their most rapid growth period, during which it might be expected that the methionine requirement would be greater than that of 170 gm. rats which are growing at a slower rate, while our results suggested a somewhat higher growth requirement for methionine in the 170 gm. rats. In addition to the different experimental techniques, another factor which made our results difficult to correlate with those of Womack and Rose was the effect of choline, which has been shown by du Vigneaud and coworkers (3) to be present in the vitamin B complex used by Womack and Rose. While it has been reported (4, 5) that choline has a growth-stimulating effect, none was found in our study with 170 gm. rats. However, it seemed possible that in 50 gm. rats there might be a methionine-sparing effect of choline as regards growth. Thus it appeared desirable to extend our observations to rats of 50 gm. initial weight. The data presented below, when considered in conjunction with the observations in 170 gm. rats and the work of Horning and Eckstein (6) with adult male rats, indicate that under certain experimental conditions the needs of the white rat for methionine, cystine, and choline and the metabolic interrelationship of these three substances change, at least quantitatively, with the age of the animal.

## EXPERIMENTAL

Young white rats weighing approximately 50 gm. (range 47 to 53 gm.) of the Sprague-Dawley strain were used. They were distributed among the dietary groups in a random manner, without regard to sex. The care of the animals has been described previously (7). The basal diet (No. 26, Tables I to III) was composed of 15.4 per cent casein, 3.2 per cent arachin, 5 per cent salt mixture (8), 2 per cent Cellu flour, 34.4 per cent glucose,<sup>1</sup> and 40 per cent lard. A commercial vitamin-free casein and arachin prepared from peanut flour<sup>2</sup> by the method of Johns and Jones (9) were used. The moisture content of the proteins was less than 1 per cent (1). The protein mixture in the basal diet provided 500 mg. of methionine and 100 mg. of cystine. The various dietary constituents supplied a total of not more than 1 mg. of choline per 100 gm. of diet. When supplementary methionine, cystine, or choline was incorporated in the basal diet as indicated in Tables I to III, an equivalent amount of glucose was omitted. All rats received orally 0.1 cc. of U. S. P. XI cod liver oil and 0.1 cc. of a solution containing 25  $\gamma$  of thiamine, 20  $\gamma$  of riboflavin, 100  $\gamma$  of calcium pantothenate, 100  $\gamma$  of nicotinic acid, and 20  $\gamma$  of pyridoxine per day. The experimental period was 21 days. The livers were removed from the animals under sodium amytal anesthesia and analyzed for total lipides (10). Apparent differences were analyzed for significance by the *t* method of Fisher (11), and only those showing a *P* value of less than 0.01 were considered significant.

*Results*

Table I summarizes the data obtained when Diet 26 was supplemented with increasing quantities of methionine. The response of the 50 gm. rats used in this study was markedly different from that observed in the 170 gm. rats used previously. First, some of the animals receiving the diets of lower methionine content (Diets 26, 27, 32) died between the 8th and 14th day of the experiment. Also a few of the animals which survived the experimental period showed a weight plateau lasting 3 to 5 days and a decrease in the food intake during the same interval. At autopsy, in the animals dying during the experimental period, the hemorrhagic kidney condition described by Griffith and Wade (4) was found. Also when the remaining animals were sacrificed at the end of the experimental period, gross inspection indicated in some of the animals the healed hemorrhagic kidneys described by Griffith (12). The occurrence of healed hemorrhagic kidneys was closely correlated with the occurrence of the weight plateau and the decrease in food intake. These phenomena were not observed in the older rats receiving these diets (1). Thus there was considerable vari-

<sup>1</sup> Generously supplied by the Corn Products Refining Company, New York.

<sup>2</sup> Proflo brand, kindly furnished by the Traders Oil Mill Company, Fort Worth.

ation among the young rats fed Diets 26, 27, and 32. However, in most of the animals showing a weight plateau and decrease in food intake in the period from the 8th to the 14th day, there was, following this, a slightly greater food intake and rate of growth than were observed in the animals not exhibiting these signs, so that at the end of the experimental period there was less individual variation than would have been expected. All animals surviving the experimental period were included in the averages shown in Table I.

The second important difference found between the young and the older rats was in the methionine requirements for growth and lipotropism. At the lower levels of methionine, no differentiation between the methionine

TABLE I

*Methionine Requirements for Growth and Lipotropism in Young Rats Receiving Choline-Free Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49 to 52 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine	Cystine	No. of rats	Food intake per day*	Gain in weight†	Liver lipides per 100 gm.	
						Moist tissue	Body weight
	mg. per 100 gm. diet	mg. per 100 gm. diet		gm.	per cent	gm.	gm.
26	500	100	21 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
27	600	100	12 (1)	5.5 ± 0.2	85.1 ± 7.7	18.3 ± 2.0	1.35 ± 0.15
32	800	100	10 (2)	5.9 ± 0.1	115.1 ± 7.1	12.0 ± 1.0	0.88 ± 0.05
33	1000	100	8	6.5 ± 0.2	140.7 ± 5.6	9.8 ± 0.4	0.61 ± 0.04
48	1300	100	18	6.3 ± 0.1	132.7 ± 4.8	8.3 ± 0.4	0.34 ± 0.01
49	1500	100	8	6.3 ± 0.2	135.0 ± 5.9	6.3 ± 0.2	0.28 ± 0.02

\* Including the standard error of the mean, calculated as follows:  $\sqrt{\Sigma d^2/(n-1)}/\sqrt{n}$ .

† The difference between the initial and final weights expressed as per cent of initial weight.

requirements for growth and lipotropism was possible. Up to the level of 1000 mg. per 100 gm. of diet, there was a progressive increase in the growth rate and a progressive decrease in the liver lipide content. These results indicate that in the young rat both growth and lipotropism participate in the utilization of the available supply of methionine up to a level of 1000 mg. of methionine. Methionine in excess of 1000 mg. further lowered the liver fat without an effect on the growth rate; at the 1500 mg. level the liver fat content was the same as when the diet contained 500 mg. of methionine and 200 mg. of choline (Table III).

The effect of supplementing Diet 26 with cystine is shown in Table II. The 100 mg. increase in cystine in Diet 29 produced a distinct increase in

the level of liver fat. The apparent difference in the growth rate of the animals on Diets 26 and 29 is not statistically significant. Cystine at the 400 mg. level in Diet 50 produced death in 84.6 per cent of the animals between the 8th and 14th days. All of these animals died with the typical symptoms of hemorrhagic kidneys. It is apparent that the basal diet used in these experiments is well adapted for the demonstration of the effect of cystine in intensifying the development of hemorrhagic kidneys. Griffith (13) has suggested that the action of cystine in increasing the level of liver fat and intensifying the development of hemorrhagic kidneys is due to an improvement in the state of nutrition (growth); *i.e.*, when dietary cystine increases the growth rate, there is a concomitant increase in the choline and methionine (labile methyl) requirements, thereby decreasing the

TABLE II

*Effect of Cystine on Growth and Lipotropism in Young Rats Receiving Choline-Free Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49.0 to 52.2 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine <small>mg. per 100 gm. diet</small>	Cystine <small>mg. per 100 gm. diet</small>	No. of rats	Food intake per day*  <small>gm.</small>	Gain in weight†  <small>per cent</small>	Liver lipides per 100 gm.	
						Moist tissue  <small>gm.</small>	Body weight  <small>gm.</small>
26	500	100	24 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
29	500	200	15 (3)	5.2 ± 0.2	69.5 ± 6.3	30.5 ± 1.1	2.73 ± 0.10
50	500	400	13 (11)	5.4	72.8	32.9	2.43

\* See Table I.

† See Table I.

amount available for lipotropic action and the prevention of hemorrhagic kidneys. Both of these effects of cystine are demonstrated in this experiment without a significant increase in the growth rate. In Paper III of this series we shall present further studies which show that the suggestion of Griffith does not explain the action of cystine on liver fat and the kidneys under all experimental conditions.

The data obtained when the basal diet was supplemented with choline are summarized in Table III. The symptoms of hemorrhagic kidneys were not observed in any of the animals receiving choline. The growth rate was increased by the choline supplements up to a level of 200 mg. per 100 gm. of diet, while the liver fat was decreased to an essentially normal value at the 100 mg. level. In our previous studies (1) with older animals, we have not observed a growth-promoting effect of choline. However, several other laboratories have reported this action of choline (14).

The results of the present study demonstrate some important differences in the response of 50 and 170 gm. rats to the same dietary régime. In 170 gm. rats receiving the basal diet used in these studies, 600 mg. of methionine per 100 gm. of diet are required for optimum growth, and in the absence of choline, an additional 600 mg. of methionine are needed to maintain a normal level of liver fat. In these older animals, hemorrhagic kidneys did not occur even on the unsupplemented basal diet. There is no significant lowering of liver fat by methionine in such animals until the growth requirement is satisfied. On the basis of present knowledge regarding the metabolic interrelationships of methionine and choline, it may be assumed that in 50 gm. rats receiving choline-free diets methionine is needed for growth and that labile methyl groups derived from methionine

TABLE III

*Choline Requirements for Growth and Lipotropism in Young White Rats*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 49.0 to 52.4 gm. The numbers in parentheses indicate the number of animals dying during the experimental period.

Diet No.	Methionine	Cystine	Choline	No. of rats	Food intake per day*	Gain in weight†	Liver lipides per 100 gm.	
							Moist tissue	Body weight
	mg. per 100 gm. diet	mg. per 100 gm. diet	mg. per 100 gm. diet		gm.	per cent	gm.	gm.
26	500	100	0	21 (6)	5.3 ± 0.1	64.2 ± 5.5	24.1 ± 1.8	2.00 ± 0.19
34	500	100	100	8	6.0 ± 0.2	88.0 ± 5.1	8.2 ± 0.4	0.40 ± 0.04
35	500	100	200	8	5.7 ± 0.1	100.4 ± 5.6	6.6 ± 0.3	0.29 ± 0.17
51	500	100	300	16	6.4 ± 0.1	95.7 ± 3.0	7.2 ± 0.4	0.32 ± 0.01

\* See Table I.

† See Table I.

are needed for lipotropism and for the prevention of hemorrhagic kidneys. The data in Table I suggest that these three requirements compete for the available supply of methionine, but that no one takes precedence over the others. For example, if one compares the results with Diet 32 to those with Diet 26, the 300 mg. of additional methionine in Diet 32 produced an increase in the growth rate, a lowering of the liver fat, and a decrease in the incidence of hemorrhagic kidneys. However, it is apparent that none of the three requirements was completely satisfied at this level of methionine. The further increase of 200 mg. of methionine in Diet 33 produced optimum growth, a complete absence of hemorrhagic kidneys, and a nearly normal level of liver fat. When the data in Tables I and III are compared, it can be concluded that the 500 mg. of methionine and 100 mg. of cystine are not sufficient to support optimum growth, for while supplementing the



diet with 200 mg. of choline (Diet 35) gave maximum lowering of the liver lipides, complete protection against hemorrhagic kidneys, and maximum stimulation of growth by choline, the growth rate was distinctly less than that found with the higher levels of methionine. Assuming that the choline in Diet 35 satisfied the requirements for lipotropism and prevention of hemorrhagic kidneys, then the 500 mg. of methionine which the diet contained were all available for growth. However, the 100 per cent increase in weight is less than optimum, as is shown by the group fed Diet 33; therefore the growth requirement for methionine in the 50 gm. rat is greater than 500 mg. when 100 mg. of cystine are present. Thus, the growth-stimulating effect of dietary choline in young rats, in contrast to the absence of such an effect in older animals, is most simply explained by a preferential utilization of methionine for growth in the older animals, so that the addition of choline does not decrease the demand on the available supply, whereas in the young animals, several metabolic processes are participating in the over-all utilization of the amino acid, and the introduction of choline into the diet supplies the needed methyl groups for at least two of these processes, thereby sparing methionine for growth.

#### SUMMARY

Young white rats (50 gm. initial weight), receiving a choline-free diet containing 18.6 gm. of protein, 500 mg. of methionine, and 100 mg. of cystine per 100 gm., grew at a rate less than optimum and developed fatty livers and hemorrhagic kidneys.

When the diet contained 1000 mg. of methionine, growth was optimum, there were no symptoms of hemorrhagic kidneys, and the level of liver fat was only slightly above normal. The total methionine requirement was between 1300 and 1500 mg. per 100 gm. of diet.

When the diet contained 200 mg. of cystine, there was a distinct antilipotropic effect without a significant increase in the growth rate. Cystine, at the 400 mg. level, produced death in 85 per cent of the animals within 14 days.

The greatest stimulation of growth by choline was observed at the 200 mg. level, which also produced a normal fat content in the liver. It is suggested that the growth-stimulating effect of choline may be attributed to a methionine-sparing action.

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## GROWTH AND LIPOTROPISM

### III. THE EFFECT OF SUPPLEMENTARY CYSTINE, METHIONINE, AND CHOLINE IN LOW PROTEIN DIETS

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(Received for publication, June 12, 1918)

Since the initial observation by Beeston and Channon (1) of the antilipotropic effect of cystine, there has been no entirely satisfactory explanation of this effect of the amino acid. Griffith (2) has explained the antilipotropic effect of cystine on the basis that supplementary cystine raises the metabolic level and thus creates an increased demand for lipotropic factors. This suggestion is attractive in that it does not involve a direct metabolic antagonism between cystine and choline or choline precursors such as methionine. Stetten and Grail (3) have shown that the antilipotropic effect of cystine does not involve an antagonism between cystine and choline. The data of Treadwell, Groothuis, and Eckstein (4) have been interpreted (5) as indicating a direct antagonism between cystine and methionine. However, this interpretation was not suggested by Treadwell *et al.*, and their data may be explained in other ways.

In Paper I of this series (6), it was shown that in 170 gm. rats receiving a choline-free basal diet, containing 18.6 per cent protein, 600 mg. per cent of methionine, and 100 mg. per cent of cystine, supplementary cystine up to a level of 600 mg. did not exhibit an antilipotropic effect or increase the growth rate. In a subsequent study (7), 50 gm. rats on the same basal diet showed an increase in liver fat and in the incidence of hemorrhagic kidneys without a significant increase in growth rate when given supplements of cystine.

In our studies of the various factors involved in the production and prevention of fatty livers, we have assumed that they will act either to increase or decrease the supply of methyl groups available for lipotropism. While the results obtained in this laboratory with supplementary cystine were not in disagreement with the suggestion of Griffith (2), it seemed desirable to extend our studies on cystine. It was decided to change the experimental conditions from those previously used so that the action of cystine on growth and lipotropism might be more clearly defined. In this connection it seemed especially desirable to avoid the development of the hemorrhagic kidney condition. As will be shown below, this was partly avoided by using animals of an initial weight of 100 gm. Rats of this weight, as shown by

Griffith (8), are more resistant to the condition than 40 gm. animals. Also a basal diet modeled after the one used by Osborne and Mendel (9) in their classical studies on the growth-promoting effect of cystine seemed preferable to the high protein diet used in our previous studies. For purposes of comparison, methionine and choline supplements were included in addition to cystine.

### EXPERIMENTAL

White rats, of both sexes, weighing approximately 100 gm. (range 97 to 104 gm.), were used. Those used in Experiment A, Table I, were of the Carworth strain; in Experiments B, C, and D (Tables I and II), the Sprague-Dawley strain was used. The care of the animals was the same as described previously (10). Two basal diets were used. Diet 100 (Table I) consisted of 5 per cent casein, 2 per cent Cellu flour, 5 per cent salt mixture (11), 48 per cent glucose,<sup>1</sup> and 40 per cent lard. Diet 112 (Table II) was like Diet 100 except that it contained 9 per cent casein and 44 per cent glucose. The supplements shown in Tables I and II were introduced at the expense of the glucose. The casein was a commercial vitamin-free product. The various dietary constituents supplied a total of less than 1 mg. of choline per 100 gm. of diet. All rats received orally 0.1 cc. of U. S. P. XI cod liver oil and 0.1 cc. of a solution containing 25  $\gamma$  of thiamine, 20  $\gamma$  of riboflavin, 100  $\gamma$  of calcium pantothenate, 100  $\gamma$  of nicotinic acid, and 20  $\gamma$  of pyridoxine per day. The experimental period was 21 days. The food intake was determined daily and the weight change were recorded three times weekly. The livers were removed from the animals under sodium amytal anesthesia and analyzed for total lipides (12). Apparent differences were evaluated by the *t* test of Fisher (13), and only those showing a *P* value of less than 0.01 were considered significant.

### Results

In Experiment A (Table I), the rats receiving Diet 100 lost 8.2 per cent of their initial body weight and developed fatty livers during the 21 day period. Diet 101 contained 100 mg. of supplementary cystine. With this diet the weight loss was the same as in those receiving the basal diet. The 100 mg. of supplementary cystine exhibited a distinct antilipotropic effect, increasing the liver fat from 18.4 to 24.5 per cent. Thus, there was an antilipotropic effect of cystine in the absence of any stimulation of growth. Diet 102 contained 100 mg. of supplementary cystine and 100 mg. of choline. On this diet the animals also lost weight. However, due to the inclusion of choline, the liver fat was decreased from 24.5 to 15.1 per cent. Diet 105 contained 124 mg. of methionine, an amount of methionine equiv-

<sup>1</sup> Generously supplied by the Corn Products Refining Company, New York.

alent to 100 mg. of cystine on the basis of sulfur. There was no stimulation of growth. Since the methionine was not used in growth, it was available for lipotropism, and there was a small decrease in the liver lipides. The 100 mg. of choline in Diet 106 lowered the liver fat to the same extent as in Diet 107. The differences in the levels of liver lipides between groups on Diets 100 and 101 and between groups on Diets 107 and 102 demonstrate

TABLE I

*Effect of Supplementary Cystine, Methionine, and Choline in Rats Receiving 5 Per Cent Casein (Choline-Free) Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 99.2 to 101.1 gm. The composition of the basal diet is described in the text.

Diet No	Supplements per 100 gm. diets			No. of rats	Food intake per day*	Change in weight†	Liver lipides per 100 gm.	
	Methionine	Cystine	Choline				Moist weight	Body weight
Experiment A								
	mg.	mg.	mg.		gm.	per cent	gm.	gm.
100	0	0	0	8	5.3 ± 0.3	-8.2 ± 1.9	18.4 ± 0.7	1.09 ± 0.09
101	0	100	0	8	5.3 ± 0.3	-7.6 ± 1.9	24.5 ± 1.6	1.81 ± 0.21
102	0	100	100	8	5.7 ± 0.2	-3.1 ± 1.4	15.1 ± 1.2	0.77 ± 0.09
105	124	0	0	7	5.4 ± 0.2	-8.1 ± 1.9	16.3 ± 0.7	0.95 ± 0.06
106	124	0	100	6	5.4 ± 0.3	-6.8 ± 2.3	11.0 ± 0.8	0.59 ± 0.08
107	0	0	100	6	6.1 ± 0.2	-9.4 ± 1.4	11.0 ± 1.2	0.50 ± 0.06
Experiment B								
100	0	0	0	8	4.3 ± 0.1	-18.8 ± 2.0	19.3 ± 1.3	1.10 ± 0.13
101	0	100	0	3	4.4	-21.2	27.9	1.70
108	0	300	0	8	4.6 ± 0.2	-14.6 ± 2.2	27.9 ± 1.6	2.02 ± 0.20
109	372	0	0	8	4.5 ± 0.2	-14.0 ± 1.3	12.3 ± 1.2	0.64 ± 0.10

\* Including the standard error of the mean, calculated as follows:  $\sqrt{\Sigma d^2/(n-1)}/\sqrt{n}$ .

† The difference between the initial and final weights expressed as per cent of initial weight.

the antilipotropic action of cystine. Of some interest in connection with the antilipotropic effect of cystine is the lowering of liver fat by choline in the different diets. With Diet 102 the liver fat was decreased from 24.5 to 15.1 per cent, a decrease of 9.4 per cent. With Diet 107 the lipides were decreased from 18.4 to 11.0 per cent, a lowering of 7.4 per cent. Thus, the effect of 100 mg. of choline was approximately the same in both diets. In the two diets containing cystine, the liver fat was at a higher level than in the corresponding diets without cystine. This may be interpreted as indi-

cating that in both diets the effect of the supplementary cystine was to lower the total supply of lipotropic methyl groups.

We had assumed on the basis of previous work (14, 15) that growth would be stimulated when Diet 100 was supplemented with the sulfur amino acids. It appeared that there were two possible explanations for the failure of methionine and cystine to stimulate growth on this basal diet. First, the supplements were too small to demonstrate their effect, or secondly, in addition to a low sulfur content there was some other limiting factor operating. In Experiment B (Table I), the first possibility was investigated. The supplements were increased to 300 mg. of cystine and 372 mg. of methionine. The rats used in Experiment B were from our laboratory colony which is of the Sprague-Dawley strain. These animals exhibited a greater weight loss on the basal diet than the rats of the Carworth strain used in Experiment A. The results with Diets 108 and 109 show that the failure to produce a stimulation in growth with the smaller supplements was not related to the low level of the supplements. This experiment also confirms the finding in Experiment A that the antilipotropic effect of cystine may be demonstrated in the absence of any stimulation of growth.

In Experiment C (Table II), the second possibility was investigated. The casein in the basal diet was increased to 9 per cent (Diet 112). On this diet rats from our colony lost weight slowly during the experimental period. Supplements of 100 mg. of cystine or 124 mg. of methionine produced a stimulation of growth. The growth rate was of the same magnitude with both amino acids. With Diet 113 there was an antilipotropic effect of cystine, with an accompanying stimulation of growth. The choline supplement in Diet 115 lowered the liver lipides to an essentially normal level, with no effect on the growth rate. A further stimulation of growth occurred at the higher levels of supplementary cystine and methionine. The results with cystine suggest that the young rat can use more than 100 mg. of cystine to supply its sulfur amino acid requirement. Actually the 300 mg. of cystine were more effective in stimulating growth during the first 12 to 14 days of Experiment C than the data indicate. The growth and food intake of the animals on Diet 110 closely paralleled those of the rats receiving Diet 111 up to the 12th and 14th day; then the food intake fell off and the body weight reached a plateau or decreased slightly. At autopsy there were no gross signs of hemorrhagic kidneys. These changes in food intake and growth were not observed in the other dietary groups or in the animals receiving Diet 110 in Experiment D.

The animals in Experiment D grew at a greater rate than the animals of Experiment C on comparable diets. The difference in response in the two experiments may have been due to the fact that the experiments were run at different seasons of the year or that because of unavoidable condi-

tions the stock diet of our animal colony was changed in the interval between Experiments C and D. However, qualitatively, the results confirm those of Experiment C. Salmon (16) has recently published data, obtained with weanling rats, on the physiological relationships of protein, fat, choline, methionine, cystine, nicotinic acid, and tryptophan. Comparison of the results of the present study with those of Salmon emphasize the importance of taking into consideration the age of the experimental animals in data

TABLE II

*Effect of Supplementary Cystine, Methionine, and Choline in Rats Receiving 9 Per Cent Casein (Choline-Free) Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 99.1 to 101.6 gm. The composition of the basal diet is described in the text.

Diet No.	Supplements per 100 gm. diets			No. of rats	Food intake per day*	Change in weight†	Liver lipides per 100 gm.	
	Methionine	Cystine	Choline				Moist weight	Body weight
Experiment C								
	mg.	mg.	mg.		gm.	per cent	gm.	gm.
112	0	0	0	8	5.5 ± 0.3	-3.6 ± 2.7	22.2 ± 1.8	1.34 ± 0.17
113	0	100	0	8	6.1 ± 0.2	+6.4 ± 1.3	26.6 ± 1.9	1.76 ± 0.21
114	124	0	0	9	6.0 ± 0.4	+5.9 ± 2.8	19.5 ± 2.4	1.02 ± 0.15
115	0	0	100	6	5.5 ± 0.3	-2.4 ± 2.1	8.6 ± 0.5	0.36 ± 0.03
110	0	300	0	11	6.0 ± 0.2	+10.4 ± 2.9	27.9 ± 1.6	2.24 ± 0.20
111	372	0	0	9	7.0 ± 0.3	+18.4 ± 2.0	14.5 ± 2.0	0.75 ± 0.15
Experiment D								
110	0	300	0	6	7.5 ± 0.5	+28.0 ± 5.7	27.0 ± 3.5	2.03 ± 0.51
111	372	0	0	6	7.5 ± 0.2	+28.3 ± 2.0	12.9 ± 1.0	0.56 ± 0.06
115	0	0	100	6	5.8 ± 0.2	+13.1 ± 2.9	7.6 ± 0.3	0.27 ± 0.01
116	0	300	100	8	6.6 ± 0.2	+24.9 ± 2.2	12.4 ± 1.0	0.52 ± 0.07
117	372	0	100	8	7.1 ± 0.3	+31.2 ± 2.3	12.3 ± 1.1	0.45 ± 0.03

\* See Table I.

† See Table I.

obtained in different laboratories. Salmon found that with weanling rats the primary deficiency in low casein diets was labile methyl groups, while in this study with 100 gm. rats, the primary deficiency was in sulfur amino acids. We have suggested previously (7) that the growth-stimulating effect of choline is most simply explained on the basis of a methionine-sparing action.

The failure of methionine and cystine to stimulate growth in rats receiving 5 per cent casein diets was unexpected, inasmuch as a stimulation has often



been reported earlier (14, 15). A review of the earlier studies suggests that this difference may be related to the use of vitamin concentrates, while in the present study, pure vitamins were administered. The concentrates possibly supplied amino acids or other factors which are limiting in a purified 5 per cent casein diet. A second possibility is that in the present study the diets contained 40 per cent fat, so that the daily food intake was less than with diets of lower calorie content.

The results of these experiments clearly show that the antilipotropic effect of cystine is not related to an increase in the nutritional level as suggested by Griffith (2). Two other explanations of the antilipotropic effect may be cited. First, in the metabolism of cystine methyl groups may be required. Secondly, the addition of cystine may decrease the rate of removal of the methyl group from methionine by a mass action effect, for, as has been convincingly shown by du Vigneaud and coworkers (17), the demethylation of methionine yields homocysteine which combines with serine to form cystathionine which is then cleaved to liberate cystine. When cystine is added, the concentration of the end-product of the above series of reactions is increased, which may decrease the rate of the reaction.

#### SUMMARY

Under the conditions of the present study, supplementary cystine and methionine did not stimulate growth when added to a 5 per cent casein diet. Growth was stimulated when these amino acids were added to a 9 per cent casein diet.

The magnitude of the growth response to supplements of cystine and methionine was approximately the same for both amino acids, except during a period of decreased food intake by the cystine-fed animals in one experiment. Amounts of cystine greater than 100 mg. per 100 gm. of diet can be utilized by the rat for growth.

Cystine can produce an antilipotropic effect without an accompanying increase in the nutritional level.

Choline produces comparable decreases in the liver lipides in the presence or absence of cystine.

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## THE VITAMIN B<sub>6</sub> GROUP

### XIV. DISTRIBUTION OF PYRIDOXAL, PYRIDOXAMINE, AND PYRIDOXINE IN SOME NATURAL PRODUCTS\*

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(Received for publication, July 1, 1918)

Although pyridoxal, pyridoxamine, and pyridoxine are all known to occur naturally (1), almost no information concerning the distribution of these individual forms of vitamin B<sub>6</sub> is available. Use of three microorganisms for the individual detection of these three substances has been previously suggested (1, 2). Since *Lactobacillus casei* responds only to pyridoxal,<sup>1</sup> this substance could be determined directly. Both pyridoxamine and pyridoxal, but not pyridoxine, promote growth of *Streptococcus faecalis*;<sup>1</sup> pyridoxamine was thus obtained as the difference between the value obtained with this organism and with *Lactobacillus casei*, corrected for the difference in activity of the two compounds for the former organism. Total vitamin B<sub>6</sub> was determined with *Saccharomyces carlsbergensis* 4228, which responds to pyridoxal, pyridoxamine, and pyridoxine. The difference between the assay value obtained with *Saccharomyces carlsbergensis* and the sum of the values for pyridoxal and pyridoxamine was considered to represent pyridoxine.

Validity of this approach to the problem was questioned when it was found that assay of yeast and liver extracts with *Streptococcus faecalis* gave higher values for their vitamin B<sub>6</sub> content than did assay with *Saccharomyces carlsbergensis* (3). Subsequently this result was shown to be due chiefly to the presence in natural materials of a "bound" form of vitamin B<sub>6</sub>, pyridoxamine phosphate, which was inactive for *Saccharomyces carlsbergensis* but active for *Streptococcus faecalis* (4). Present availability of improved hydrolytic procedure for release of vitamin B<sub>6</sub> from tissues (5)

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

<sup>1</sup> At high levels, addition of either pyridoxine or pyridoxamine to the medium permits growth of *Lactobacillus casei*; they are less than 0.001 as active as pyridoxal for this organism, however, and their contribution to the analytical values found for natural materials is entirely negligible (2). Similarly, pyridoxine is less than 0.001 as active as pyridoxamine or pyridoxal for *Streptococcus faecalis*, and its contribution to analytical values obtained with the latter organism is nil.

and of improved assay methods with *Streptococcus faecalis* (6) and *Lactobacillus casei* (7) permitted reexamination of the utility of a differential assay procedure for the various forms of vitamin B<sub>6</sub>. Results of this study are presented below.

#### EXPERIMENTAL

**Assay Procedures**—Pyridoxal was determined with *Lactobacillus casei* (7); *Streptococcus faecalis* R was used for determination of pyridoxal plus pyridoxamine (6). *Saccharomyces carlsbergensis* 4228 was used (5, 8) for

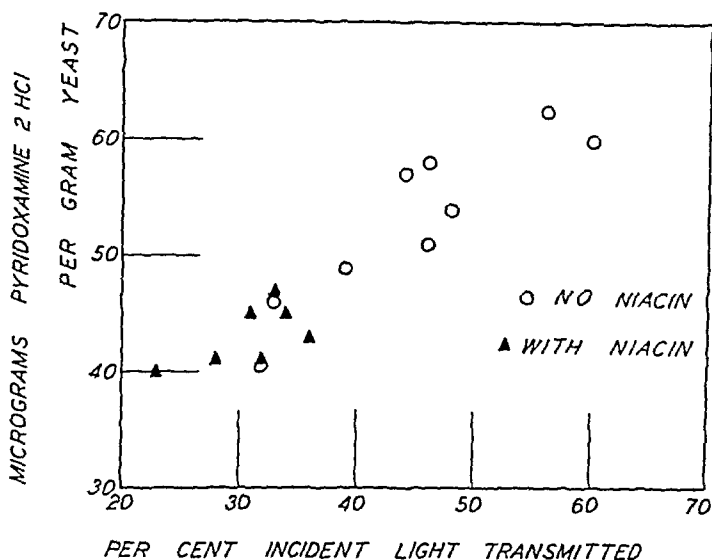


FIG. 1. The effect of nicotinic acid on the determination of vitamin B<sub>6</sub> with *Saccharomyces carlsbergensis*. Abscissa, per cent of incident light transmitted by a 20 hour culture of *Saccharomyces carlsbergensis* grown in the presence of 20 mγ of pyridoxamine dihydrochloride per 5 cc. Ordinate, vitamin B<sub>6</sub> content of a dried yeast sample calculated as micrograms of pyridoxamine dihydrochloride per gm. of yeast.

the determination of total vitamin B<sub>6</sub> (pyridoxal, pyridoxamine, and pyridoxine). The basal medium used in the latter procedure has been criticized (9) for the omission of nicotinic acid. In a single experiment, no difference in the apparent vitamin B<sub>6</sub> content of a yeast sample was noted, whether or not nicotinic acid was added to the medium. However, a review of all assays conducted on this sample showed that, when nicotinic acid had been omitted from the medium, growth was less with suboptimal levels of vitamin B<sub>6</sub> than when nicotinic acid was present in the medium. With heavier growth, assay values for vitamin B<sub>6</sub> tended to be lower. These results, summarized in Fig. 1, indicate that in some but not all assays nicotinic acid

does become a growth-limiting factor in the original assay medium (8), and that under such conditions values found for vitamin B<sub>6</sub> may be high. For these reasons, nicotinic acid was included in the assay medium at a level of 0.5 mg. per 100 cc., as recommended by Hopkins and Pennington (9).

Whenever natural materials were assayed, an amount of sample estimated to contain 2  $\gamma$  of vitamin B<sub>6</sub> was finely ground, placed in 180 cc. of 0.055 N hydrochloric acid, autoclaved for 5 hours at 20 pounds pressure (5), cooled, and neutralized with potassium hydroxide solution. Samples in which solid materials remained were filtered before assay.

*Assay of Known Mixtures*—Results of assay of mixtures of pyridoxal hydrochloride, pyridoxamine dihydrochloride, and pyridoxine hydrochloride are shown in Table I. The method for calculating the amount of

TABLE I

*Determination of Pyridoxal, Pyridoxamine, and Pyridoxine in Mixtures of Known Composition*

The values are expressed as millimicrograms per cc.

Sample No.	Total assay value					Amount found			Amount added		
	<i>L. casei</i>   <i>S. faecalis</i>   <i>S. carlsbergensis</i>										
	Standard used										
	Pyri- doxal HCl (A)	Pyri- doxal HCl (B)	Pyri- dox- amine 2HCl (C)	Pyri- doxal HCl or pyridoxine HCl (D)	Pyri- dox- amine 2HCl (E)	Pyri- doxal HCl (A)	Pyri- dox- amine 2HCl (F)	Pyri- doxine HCl (G)	Pyri- doxal HCl	Pyri- dox- amine 2HCl	Pyri- doxine HCl
1	0.41	0.88	0.84	1.20	1.48	0.41	0.45	0.43	0.40	0.40	0.40
2	0.91	1.45	1.80	1.68	2.28	0.91	0.67	0.27	0.90	0.60	0.30

each component of the mixture from the assay values requires some explanation. The dose-response curves of *Saccharomyces carlsbergensis* to pyridoxal and to pyridoxine are identical. However, the dose-response curves of both this organism and *Streptococcus faecalis* to pyridoxal and to pyridoxamine were dissimilar; i.e. the activity of pyridoxamine in terms of pyridoxal was not constant over the assay range used. Consequently, no constant factor for converting pyridoxal to its equivalent of pyridoxamine (or vice versa) could be used. To meet this situation, standard dose-response curves to each compound were obtained with each organism. The vitamin B<sub>6</sub> content of the samples was then calculated in terms of each of these standards (Columns B to E, Table I). The value found in terms of pyridoxamine divided by the value found in terms of pyridoxal thus gives a ratio which expresses the "average" activity of pyridoxal in terms of pyridoxamine over that portion of the curve used in calculating the results.

The individual components of the mixture are then calculated as follows:

$$\text{Pyridoxal hydrochloride} = A$$

$$\text{Pyridoxamine dihydrochloride} = C - \frac{C}{B} \times A = F$$

$$\text{Pyridoxine hydrochloride} = D - (A + F \times \frac{D}{E}) = G$$

where  $A$ ,  $B$ , etc., are the values found with the individual assay organisms against the appropriate standard, as indicated in Tables I to IV. The validity of this method is shown by the results obtained in the analysis of the two mixtures (Table I). Recoveries of pyridoxal hydrochloride were 102 and 102 per cent, of pyridoxamine dihydrochloride 112 and 112 per cent, and of pyridoxine hydrochloride 90 and 108 per cent of the amounts added in the two samples.

The limitations of a differential assay procedure of this type should be emphasized. If one accepts  $\pm 10$  per cent as the approximate limits within which values found by each of the three assay methods are reproducible (see below), then it is apparent that a variation of  $\pm 10$  per cent of the total assay value found with *Streptococcus faecalis* (pyridoxal plus pyridoxamine) might be expected in the value calculated for pyridoxamine alone. Similarly, the variation encountered in the calculated value for pyridoxine will be  $\pm 10$  per cent of the total assay value found with *Saccharomyces carlsbergensis*, which responds to pyridoxal, pyridoxamine, and pyridoxine. It is thus clear that values for pyridoxal are relatively accurate, those for pyridoxamine less so, and those for pyridoxine are least so. The credibility of values for pyridoxine, for example, will depend upon the proportion of the total vitamin B<sub>6</sub> which it represents. If this is only 10 per cent or less of the total assay value with *Saccharomyces carlsbergensis*, the value calculated for it is clearly meaningless.

A more accurate estimate of the validity of these assays may be gained from Table II. Here the results of six wholly independent analyses of a dried yeast are shown. The coefficients of error in the determination of vitamin B<sub>6</sub> with *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* were 6.3, 8.0, and 4.2 per cent, respectively, of the mean value. The pyridoxal, pyridoxamine, and pyridoxine contents of the yeast and the probable error of each value were calculated from these average figures and their standard errors by the method previously described. From the tabulated results it is apparent that, while the values for pyridoxal and pyridoxamine are highly significant, those for pyridoxine are not, and it cannot be said with certainty whether or not any pyridoxine is present. It is certain, however, that the amount of pyridoxine present, if any, is comparatively small.

The error of the mean value, calculated on the assumption that the total

assay value as determined with each organism is subject to 10 per cent error in either direction, is shown in the last line of Table II. Examination of the individual assay values shows that most, though not all of these, fall between these limits. Consequently, this procedure has been used in Tables III and IV to provide a rough measure of the probable degree of accuracy of the single assay values recorded. Although in some cases errors larger than those indicated can be expected, the satisfactory values

TABLE II  
Vitamin B<sub>6</sub> Content of Dried Yeast

The values are expressed in micrograms per gm.

Trial No.	Total assay value					Calculated content		
	<i>L. casei</i>	<i>S. faecalis</i>	<i>S. carlsbergensis</i>					
	Standard used							
	Pyridoxal HCl (A)	Pyridoxal HCl (B)	Pyridox- amine 2HCl (C)	Pyridoxal HCl or pyridox- amine HCl (D)	Pyridox- amine 2HCl (E)	Pyridoxal HCl (A)	Pyridox- amine 2HCl (F)	Pyri- doxine HCl (G)
1	4.9	42	41	34	41	4.9	36	-0.8
2	6.1	50	49	35	43	6.1	43	-6.3
3	4.1	56	53	37	45	4.1	49	-7.3
4	5.1	57	46	37	45	5.1	42	-2.9
5	6.0	42	42	38	46	6.0	36	2.2
6	4.9	54	44	40	51	4.9	40	3.7
Average $\pm$ s.e.m.*	5.2 $\pm 0.33$	50 $\pm 4.0$	46 $\pm 2.7$	38 $\pm 1.4$	45 $\pm 1.9$	5.2 $\pm 0.33$	41 $\pm 2.9$	-1.9 $\pm 1.9$
Average $\pm 10\%$ total assay value.	5.2 $\pm 0.5$		46 $\pm 4.6$		45 $\pm 4.5$	5.2 $\pm 0.5$	41 $\pm 5.0$	-2 $\pm 4.5$

\* Standard error of mean =  $\sqrt{(\Sigma d_a^2)/(n(n-1))}$  where  $d_a$  is the deviation of a particular observation from the arithmetic mean and  $n$  is the number of observations (10).

obtained in recovery experiments (Tables I and III) show that frequently the values obtained are much more accurate than indicated.

*Recoveries of Known Mixtures from Natural Samples*—To test further the assay procedure, known mixtures of pyridoxal, pyridoxamine, and pyridoxine were added to natural materials which were then hydrolyzed as described above and assayed. Results were calculated as described earlier, and are given, together with their possible variation, in Table III. With some exceptions, recoveries are surprisingly good, even when the amounts added are small in comparison to the total assay figure. In a few cases,



TABLE III  
Recovery of Mixtures of Pyridoxal, Pyridoxamine, and Pyridoxine Added  
to Natural Products

Sample	Total assay value					Content			Amount recovered		
	<i>L. casei</i>	<i>S. faecalis</i>	<i>S. carlsbergensis</i>								
	Standard used										
	Pyri- doxal HCl  (A)	Pyri- doxal HCl  (B)	Pyri- dox- amine 2HCl  (C)	Pyri- doxal HCl or pyri- doxine HCl  (D)	Pyri- dox- amine 2HCl  (E)	Pyri- doxal HCl  (A)	Pyri- dox- amine 2HCl  (F)	Pyri- doxine HCl  (G)	Pyri- doxal HCl  (A)	Pyri- dox- amine 2HCl  (B)	Pyri- doxine HCl  (C)
$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	
Wilson 1:20 liver powder	9.8	46	30	48	58	9.8 $\pm 1.0$	24 $\pm 5$	18 $\pm 6$			
Wilson 1:20 liver powder + added vitamin B <sub>6</sub>	19	148	102	221	268	19 $\pm 2$	89 $\pm 15$	128 $\pm 27$	9 $\pm 2$	65 $\pm 15$	110 $\pm 27$
Added vitamin B <sub>6</sub>									10	60	100
Cerogras	2.7	7.3	6.8	12	16	2.7 $\pm 0.3$	4.3 $\pm 0.7$	6 $\pm 2$			
“ + added vitamin B <sub>6</sub>	8.1	26	21	54	67	8.1 $\pm 0.8$	14 $\pm 3$	35 $\pm 7$	5.4 $\pm 0.8$	10 $\pm 3$	29 $\pm 7$
Added vitamin B <sub>6</sub>									5	10	25
Dried Yeast I	5.1	57	46	37	45	5.1 $\pm 1$	42 $\pm 6$	-3 $\pm 5$			
“ “ I + added vitamin B <sub>6</sub>	18	90	69	74	88	18 $\pm 2$	55 $\pm 9$	10 $\pm 9$	13 $\pm 2$	13 $\pm 9$	10 $\pm 9$
Added vitamin B <sub>6</sub>									12	12	12
Dried Yeast II	4.9	54	44	40	51	4.9 $\pm 0.5$	40 $\pm 5$	4 $\pm 5$			
“ “ II + added vita- min B <sub>6</sub>	9.7	144	121	216	278	9.7 $\pm 1.0$	113 $\pm 14$	118 $\pm 28$	4.6 $\pm 1.0$	73 $\pm 14$	114 $\pm 28$
Added vitamin B <sub>6</sub>									5	50	100
Urine	100	293	258	190	242	100 $\pm 10$	170 $\pm 29$	-43 $\pm 24$			
“ + added vitamin B <sub>6</sub>	199	678	562	772	953	199 $\pm 20$	397 $\pm 68$	253 $\pm 95$	99 $\pm 20$	227 $\pm 68$	253 $\pm 95$
Added vitamin B <sub>6</sub>									100	200	400

e.g. pyridoxamine recovery from Dried Yeast II, recoveries were less accurate than were expected.

TABLE IV  
Pyridoxal, Pyridoxamine, and Pyridoxine Content of Some Natural Materials

Sample	Pyridoxal HCl	Pyridoxamine 2HCl	Pyridoxine HCl
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Chick liver.....	38 $\pm$ 4	46 $\pm$ 8	0 $\pm$ 10
" heart.....	8.0 $\pm$ 0.8	12 $\pm$ 2	-1 $\pm$ 2
" breast muscle.....	37 $\pm$ 4	1 $\pm$ 4	8 $\pm$ 6
" brain.....	11 $\pm$ 1	7 $\pm$ 2	3 $\pm$ 3
" kidney.....	30 $\pm$ 3	26 $\pm$ 6	-3 $\pm$ 6
Rat liver.....	29 $\pm$ 3	9 $\pm$ 4	9 $\pm$ 5
" heart.....	16 $\pm$ 2	9 $\pm$ 3	1 $\pm$ 3
" breast muscle.....	22 $\pm$ 2	-1 $\pm$ 2	5 $\pm$ 3
" brain.....	9.2 $\pm$ 0.9	4 $\pm$ 3	4 $\pm$ 2
" kidney.....	33 $\pm$ 3	13 $\pm$ 5	10 $\pm$ 6
" spleen.....	3.8 $\pm$ 0.4	0.7 $\pm$ 0.4	-0.4 $\pm$ 0.5
" sarcoma.....	2.3 $\pm$ 0.2	9 $\pm$ 1	3 $\pm$ 2
Fresh beef liver.....	7.0 $\pm$ 0.7	31 $\pm$ 4	-3 $\pm$ 4
Wilson 1:20 liver powder.....	6.1 $\pm$ 0.6	31 $\pm$ 4	15 $\pm$ 5
Liver concentrate, Sharp and Dohme	16 $\pm$ 2	47 $\pm$ 8	6 $\pm$ 15
Frozen fish.....	10 $\pm$ 1	22 $\pm$ 3	-1 $\pm$ 3
Dried yeast, brewers'.....	4.9 $\pm$ 0.5	36 $\pm$ 4	-1 $\pm$ 4
Fresh " bakers'.....	7.5 $\pm$ 0.8	11 $\pm$ 2	0 $\pm$ 2
Dried <i>Penicillium</i> mycelium.....	3.5 $\pm$ 0.4	9 $\pm$ 1	4 $\pm$ 2
Fresh whole milk, $\gamma$ per cc.....	0.32 $\pm$ 0.03	0.09 $\pm$ 0.09	-0.02 $\pm$ 0.05
" egg white.....	0.19 $\pm$ 0.02	0.14 $\pm$ 0.03	0.39 $\pm$ 0.08
" " yolk.....	11 $\pm$ 1	4 $\pm$ 2	0 $\pm$ 2
" whole egg.....	5.6 $\pm$ 0.6	1.2 $\pm$ 0.7	0 $\pm$ 0.9
Cerogras.....	4.1 $\pm$ 0.4	2.5 $\pm$ 0.7	4.0 $\pm$ 1.0
Fresh celery.....	7.8 $\pm$ 0.8	2.5 $\pm$ 1.1	9 $\pm$ 2
" leaf lettuce.....	12 $\pm$ 1	-1 $\pm$ 2	9 $\pm$ 2
" green pepper.....	8.2 $\pm$ 0.8	53 $\pm$ 6	20 $\pm$ 8
Whole lemon.....	3.0 $\pm$ 0.3	0.7 $\pm$ 0.4	10 $\pm$ 2
Fresh carrot.....	2.1 $\pm$ 0.2	0.3 $\pm$ 0.3	7 $\pm$ 1
Whole wheat.....	1.9 $\pm$ 0.2	3.7 $\pm$ 0.4	9 $\pm$ 2
Wheat germ.....	0.87 $\pm$ 0.09	0.7 $\pm$ 0.2	3.1 $\pm$ 0.7
Split peas.....	0.47 $\pm$ 0.05	0.43 $\pm$ 0.1	1.3 $\pm$ 0.3
Yellow corn-meal.....	1.4 $\pm$ 0.1	1.1 $\pm$ 0.2	0 $\pm$ 0.3
Vitab.....	1.9 $\pm$ 0.2	10 $\pm$ 2	79 $\pm$ 11

When the method was applied to human urine, a negative value for pyridoxine was found whose magnitude was considerably greater than the expected error, even if it were assumed that no pyridoxine was present. In this case, recoveries of added pyridoxal and pyridoxamine were within

experimental error, while recovery of pyridoxine was low. These results indicate the presence in human urine of materials, toxic for *Saccharomyces carlsbergensis*, which suppress the growth response of this organism to vitamin B<sub>6</sub>, but which do not affect the responses of *Streptococcus faecalis* or *Lactobacillus casei*.

*Distribution of Pyridoxal, Pyridoxamine, and Pyridoxine in Natural Materials*—Results of assay of a variety of tissues and other natural materials are given in Table IV. The vegetables, fish, meat, and dairy products were purchased in a local grocery. Chick and rat tissues were from experimental animals on stock rations, and were prepared for analysis immediately following death from decapitation or asphyxiation. Tissues from four to six animals were pooled for assay. After homogenization in the Waring blender, separate samples were taken for hydrolysis and for dry weight determinations. Values are expressed in terms of micrograms per gm. of dry material.

Over 80 per cent of the vitamin B<sub>6</sub> in all fresh animal tissues studied was present as pyridoxal and pyridoxamine. In most of these samples, pyridoxal was the predominant form. In most cases, it was not possible to demonstrate the presence of any pyridoxine; if present at all, it constitutes only a minor fraction of the total vitamin B<sub>6</sub> of these tissues. With the exception of the fresh rat liver, pyridoxamine was the predominant form of the vitamin in all liver samples analyzed. Pyridoxal and pyridoxamine were also the predominant forms of vitamin B<sub>6</sub> in frozen fish, fresh and dried yeast, *Penicillium* mycelium, milk, and eggs. Although pyridoxal and pyridoxamine were also prominent in the plant products tested, it was evident that pyridoxine was also present to a very significant extent. In this respect, the contrast between plant and animal products is quite clear. Vitab, an extract of rice bran, is very high in pyridoxine and low in pyridoxal and pyridoxamine. It is significant, therefore, that the only procedures for isolation of pyridoxine which have been described in detail utilized rice bran as a starting material.

#### DISCUSSION

Aside from microbiological procedures, only two methods, both physico-chemical, have been suggested for determination of pyridoxal, pyridoxamine, and pyridoxine. Neither of these has been adapted to assay of natural materials.

The reaction of pyridoxine with diazotized sulfanilic acid was first considered as an analytical method for the determination of pyridoxine by Kuhn and Low (11). Ormsby, Fisher, and Schlenk (12) have shown that pyridoxal, pyridoxamine, and pyridoxine form derivatives with this reagent of differing colors, which can be differentiated spectroscopically. This reac-

tion was used to demonstrate presence of both pyridoxal and pyridoxamine in a purified preparation of transaminase. Hopkins and Pennington (9), however, noted that the colors produced with this reagent were transitory and lacked specificity.

A method for determining pyridoxine in the presence of pyridoxal and pyridoxamine has been developed by Melnick *et al.* (3). This method is based on a reaction, first noted by Stiller, Keresztesy, and Stevens (13), between pyridoxine and 2,6-dichloroquinone chloroimide, later modified by Scudi, Bastedo, and Webb (14). The reaction does not offer a means of differentiating pyridoxal from pyridoxamine, however, and its specificity when applied to natural materials is not known.

Although satisfactory in principle, the method described in this paper has severe limitations which should be recognized. Since pyridoxamine and pyridoxine are not determined directly, but by difference, the error involved in their determination is largely dependent on the relative proportion of these substances to the total vitamin B<sub>6</sub> of the sample. With the present method, it is not possible to demonstrate with certainty the presence of pyridoxine in a sample when less than 10 per cent of the total vitamin B<sub>6</sub> is pyridoxine, and errors are large even when more than this is present. This was the case with all animal tissues investigated. Likewise the present method does not allow the determination of pyridoxamine in a sample in which the pyridoxamine constitutes less than 10 per cent of the sum of the pyridoxal and pyridoxamine of the sample. Since pyridoxal is determined directly, its determination is quite satisfactory.<sup>2</sup> Unfortunately, no microorganisms are known which respond specifically to pyridoxamine or pyridoxine, so that their direct determination is not possible. A direct determination of pyridoxamine could be obtained, however, by quantitatively destroying pyridoxal with acetone and alkali (1) and assaying the resulting mixture for pyridoxamine with *Streptococcus faecalis*. Assay of the same mixture with yeast would then provide a differential determination of pyridoxine involving only two instead of three microor-

<sup>2</sup> The loose condensation products (Schiff's bases?), which pyridoxal forms with amino acids and other amines, and the thiazolidine derivative formed with cysteine (15, 16) may occur naturally. These products readily decompose to form pyridoxal, and like pyridoxal are fully active for *Lactobacillus casei* (15). It has been emphasized (16) that the transamination reaction between pyridoxal and amino acids, previously shown to occur when solutions containing these substances are heated (2, 17), occurs also to some extent in the cold, and that figures for the pyridoxal content of natural materials may hence be of doubtful significance. Previous work has shown (7), however, that this reaction does not occur under the hydrolytic conditions employed in this investigation. It is also minimized or completely avoided even at neutrality by use of dilute solutions. The analytical values given for fresh tissues, therefore, should reflect accurately the amount of pyridoxal actually present.

ganisms, which should be somewhat more accurate than the method used here. This refinement of the present procedure has not been generally applied as yet. For reliable microbiological estimates of the small amounts of pyridoxine present in animal and some other tissues, however, some procedure for its separation from pyridoxal and pyridoxamine, such as that recently suggested by Winsten and Eigen (16), will be required, so that it can be estimated separately.

The present findings confirm and expand previous indications (1, 6) that pyridoxine, if present at all, constitutes quantitatively a very minor portion of the vitamin B<sub>6</sub> of animal tissues and many other products. Despite this fact, and the fact that by no previously applied method of analysis can the various forms of this vitamin be distinguished, numerous workers continue to refer misleadingly and erroneously to the "pyridoxine" content of natural materials. If by *pyridoxine* is meant the specific chemical compound to which this name was given (18), it is evident that the pyridoxine content of such materials is unknown and is not determined by the methods used. If, on the other hand, *pyridoxine* is used as a group name to include all substances curative of vitamin B<sub>6</sub> deficiency in animals (including pyridoxal and pyridoxamine (2, 19, 20)), then it obviously should not be used also as the name for the specific compound, 2-methyl-3-hydroxy-4,5-bis-(hydroxymethyl)pyridine.

The present confusion in nomenclature results from retention of the name "pyridoxine" as synonymous with "vitamin B<sub>6</sub>" and at the same time as a name for a specific compound. A more rational and less confusing notation would result if the term "pyridoxine" were reserved only for the specific compound and the term "vitamin B<sub>6</sub>" were used as a group name to include all three compounds.

#### SUMMARY

A method for the differential determination of pyridoxal, pyridoxamine, and pyridoxine in natural products is described. This method takes advantage of differences in the specificity of response of *Lactobacillus casei*, *Streptococcus faecalis*, and *Saccharomyces carlsbergensis* to the different forms of vitamin B<sub>6</sub>. Although the principle of this method was suggested previously (2), its application to the analysis of natural materials was possible only after further development of the three individual assay procedures involved and a clarification of the hydrolytic procedures used in the liberation of the vitamins from natural materials, where they occur largely in more complex forms unavailable to the microorganisms.

Analysis of known mixtures of the three compounds by the method described gave satisfactory results. With few exceptions, satisfactory recoveries of the vitamins from natural materials were obtained. A significant

exception occurred in the case of the recovery from urine, in which the amount of pyridoxine recovered was lower than could be accounted for by analytical errors. Limitations common to differential methods of this type are discussed. It is shown that the accuracy of the figures for pyridoxine depends upon the relative proportion of this substance to pyridoxal and pyridoxamine. When pyridoxine comprises less than 10 per cent of the total vitamin B<sub>6</sub>, it cannot be determined by this procedure.

Analytical values for the pyridoxal, pyridoxamine, and pyridoxine content of natural materials are given. Pyridoxal and pyridoxamine were the predominant forms of vitamin B<sub>6</sub> in hydrolyzed animal tissues and yeasts, with only slight indications or none at all of the presence of pyridoxine in these samples. Pyridoxine was more evident in plant materials, in which it occurred in amounts as large or larger than those of pyridoxal and pyridoxamine.

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# A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF ADENINE\*

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(Received for publication, July 19, 1948)

In spite of marked advances and high interest in purine and nucleic acid biochemistry in recent years (7), adequate methods in quantitative purine chemistry are still not available. At present there is no method suitable for the accurate estimation of adenine in mixtures of purines; a similar situation exists for the other common purines, with the exception of uric acid.<sup>1</sup> The newly proposed chromatographic (9) and counter-current distribution (8) procedures, as well as the method of differential spectrophotometry (5), may prove valuable for the determination of purines in mixtures, but their usefulness has not as yet been demonstrated. The proposed use of a mutant strain of *Neurospora crassa* has been shown to have little value for the estimation of purines in mixtures (6). Thus, the report that *Clostridium acidiurici* is able to bring about a fairly complete decomposition of guanine, xanthine, and hypoxanthine, but not of adenine (1), led to the suggestion that this highly specific bacterium may be useful for the quantitative estimation of this purine.

This paper reports progress that has been made in developing a specific microbiological method for the estimation of adenine. It is shown that highly accurate adenine determinations in purine mixtures are possible by the use of *Clostridium acidiurici*. The purine content of a commercial sample of yeast nucleic acid (ribonucleic acid) is also reported.

## Methods and Materials

Heavy cell suspensions of *Clostridium acidiurici* (strain 9a) were prepared as previously described (1) with the exception that a medium containing 0.2 per cent uric acid and 0.02 per cent hypoxanthine was used for growth of the organisms. Cells grown on this medium have a higher activity towards hypoxanthine than those grown on uric acid alone. Substrate decompositions were carried out in small evacuated reaction vessels or

\* Authorized for publication on July 12, 1948, as paper No. 1452 in the Journal series of the Pennsylvania Agricultural Experiment Station. This work was supported in part by a research grant from the National Cancer Institute of the National Institute of Health, United States Public Health Service.

<sup>1</sup> Since adequate methods for uric acid are available and also because it is readily removed from biological systems by action of uricase, it has not been considered in this paper.



Thunberg tubes. The total volume of cells plus substrate ranged from 4 to 6 ml. Blank determinations on the substrate and cell suspension incubated separately were carried out and the indicated corrections were made to the analytical data.

Ammonia, acetic acid, and carbon dioxide were determined as previously outlined. Acetic acid was identified by Duclaux distillation.

Yeast nucleic acid (YNA) was hydrolyzed by the method of Graff and Maculla (2). It was found that complete liberation without decomposition of the purines was accomplished by heating at 100° with about 1 N HCl over a rather wide period of time (45 minutes to 2 hours). 1 gm. of YNA was dissolved in 10 ml. of 1 N HCl, heated to boiling for 1 hour, cooled, neutralized, and diluted to a volume of 100 ml. Aliquot portions of this solution were taken for further work.

Free purines were isolated by the method of Hitchings and Fiske (4). This method is fast, and quantitative recovery (98 to 100 per cent) of free purines, whether present singly or in mixtures, is regularly attained.

The purines used were obtained from commercial sources and were found to contain 98 to 99 per cent of the theoretical N content. The yeast nucleic acid, also a commercial sample, had a nitrogen content of only 92 per cent of the theoretical value, based on the polytetranucleotide formula. No attempt was made to purify this material further.

#### EXPERIMENTAL

*Clostridium acidilurici* (strain 9a) was shown (1) to be able to carry out a fairly complete decomposition of xanthine, guanine, and hypoxanthine, but to have little or no action on adenine. Work on these purine decompositions has yielded data which are summarized in Table I. Ammonia, carbon dioxide, and acetic acid are shown to account for 91 to 97 per cent of the carbon and nitrogen of the purines decomposed. The values shown are averages of a number of determinations made over a period of several years. The range of values is given to indicate the variation which may be expected in these determinations. The experimental error in the ammonia determinations as carried out in this work is seen to be about  $\pm 2$  per cent, for carbon dioxide about  $\pm 4$  per cent, and somewhat larger,  $\pm 8$  to 9 per cent, for acetic acid. The conversion of purine N to  $\text{NH}_3\text{-N}$  is seen to vary from 93 to 97 per cent, with an average of 95 per cent.

Ammonia production from adenine has not been detected<sup>\*</sup> in these experiments. There is some volatile acid produced from adenine; although no attempt has been made to identify this acid, it may be expected to consist of acetic acid.

<sup>\*</sup> Reported  $\text{NH}_3$  production from adenine (1) has not been found in these more recent experiments.

Since these experiments show a nearly complete and constant conversion of xanthine, guanine, or hypoxanthine nitrogen to  $\text{NH}_3\text{-N}$ , while no such reaction occurs with adenine, ammonia production by *Clostridium acidurici* from mixtures of purines may be expected only from the former three

TABLE I

*Products of Action of Cell Suspensions of Clostridium acidurici on Purines*  
The average values shown are from five or more analyses.

Substrate*	Production per 100 moles substrate			Per cent recovery	
	Acetic acid	Carbon dioxide	Ammonia	Nitrogen	Carbon
	moles	moles	moles		
Xanthine, average.....	89	279	380	95	91
" range.....	88-96	276-286	368-384	92-96	90-96
Guanine, average.....	81	286	485	97	91
" range.....	79-89	279-303	468-490	95-98	87-96
Hypoxanthine, average.....	116	225	371	93	91
" range.....	111-120	220-228	360-376	90-94	88-94
Adenine, average.....	9†	0	0		

\* Quantities of substrate varied from 0.5 to 3 mg. of purine N.

† Not identified as acetic acid.

TABLE II

*Determination of Adenine Content of Mixtures of Purines*

4 ml. of substrate, 1 ml. of cell suspension, 1 ml. of phosphate buffer, pH 7.2. Incubated 14 hours at 35° in evacuated Thunberg tube.

Tube No.	Purine nitrogen taken				Ammonia N found*	Adenine N, by difference	Adenine recovered
	Xanthine	Guanine	Hypoxanthine	Adenine			
	mg.	mg.	mg.	mg.		mg.	per cent
1	0.439	0.521	0.395	0.563	1.356	0.562	100
2	0.439	0.521	0.395	0.563	1.345	0.573	102
3	0.439	0.521	0.395	0	1.358	-0.003	
4	0.439	0	0.395	0.563	0.821	0.576	102
5	0	0.521	0.395	0.563	0.914	0.555	98
6	0.439	0.521	0.790	0	1.760	-0.010	

\* Corrected for average of 95 per cent recovery from purines.

purines. A number of experiments have been carried on which demonstrate this to be the case.

The results of one such experiment are summarized in Table II. A cell suspension of *Clostridium acidurici* was allowed to act on a solution containing the indicated mixture of purines at pH 7.2 for 14 hours at 35°.

Ammonia production was then determined. The ammonia production shown is a corrected value obtained by dividing the actual ammonia found by the factor 0.95, which is the average value for conversion of purine N shown in Table I. This introduces some slight error in those cases in which the decomposable purines are not present in approximately equal amounts. Adenine N is then calculated by subtracting the corrected  $\text{NH}_3\text{-N}$  from the total purine N, which is known in this experiment, or which may be determined experimentally as will be shown later. The data show that the adenine content of the mixtures as calculated from analytical values agrees satisfactorily with the known amount of adenine taken. The percentage recovery in the data presented here is 98 to 102 per cent, which is about the experimental error for the ammonia determination shown in Table I. Other data indicate that an accuracy of  $\pm 3$  per cent may be expected.

TABLE III

*Analysis of Purine Content of Yeast Nucleic Acid*

The values are per cent of yeast nucleic acid.

	Total N	Purine N	Guanine N (non-adenine purine N)		Adenine N, by difference
			Purines not isolated	Purines isolated	
Theoretical values . . . . .	16.55	10.06*	5.03*	5.03*	5.03*
Determined " . . . . .	15.09	9.94	5.06	4.95	4.99

\* Based on yeast nucleic acid N content of 15.09 per cent.

It is obvious that application of this procedure to complex biological material depends on a separation of the purines from other nitrogenous substances in order to arrive at a true value of the purine N. A separation is necessary also because it has been shown that glycine and perhaps other nitrogenous compounds are decomposed by *Clostridium acidurici* with ammonia production in the presence of purines (1). The method of Hitchings and Fiske has been found to be a satisfactory means of separating free purines and of producing a solution of the purines suitable for decomposition by cell suspensions of *Clostridium acidurici*.

The data presented in Table III illustrate the use of cell suspensions of *Clostridium acidurici* in the determination of the purine content of yeast nucleic acid. Total N was determined on an aliquot of the unhydrolyzed sample. Free purines were isolated from the hydrolyzed sample, and subjected to total N determination and to action of cell suspensions of *Clostridium acidurici*. The hydrolyzed sample was also treated with cell suspensions before isolation of the free purines. Ammonia N was determined on the samples subjected to action of the bacterial cells.

The results show that the purine N is very nearly that expected from consideration of the total N content of 15.09 per cent and the theoretical polytetranucleotide formula for yeast nucleic (ribonucleic) acid. The total purine N determined on the isolated purine fraction is found to be 9.94 per cent, as compared with a theoretical value of 10.06 per cent. The adenine N content measured by the non-decomposable purine N is shown to be 4.99 per cent as compared with a theoretical value of 5.03 per cent. The decomposable purine N, presumably guanine as will be shown later, is 4.95 per cent, theoretical value 5.03 per cent. A similar value for guanine N in the hydrolyzed but not separated purine fraction is seen to be 5.06 per cent. The series of values reported in Table III are from a single analysis. However, almost identical values have been obtained in three additional complete analyses as well as several other partially complete analyses.

TABLE IV

*Identification of Decomposable Portion of Yeast Nucleic Acid*

The values are averages of five or more analyses; the range of values is shown in parentheses.

Substrate	Carbon dioxide produced*	Acetic acid produced*
Hydrolyzed YNA, purines not isolated	57 (53-62)	19 (18-21)
Purines isolated from YNA . . . . .	56 (55-57)	16 (15-17)
Guanine†. . . . .	59	17
Xanthine†.....	73	23
Hypoxanthine†....	61	31

\* Based on  $\text{NH}_3$  production as 100.

† Calculated from average values shown in Table I.

Identification of the decomposable purine fraction is made possible by consideration of the quantities of acetic acid and carbon dioxide formed during the dissimilation process. The values for acetic acid and carbon dioxide production from known samples of the three labile purines are shown in Table IV calculated to the basis of ammonia production equal to 100 from the data in Table I. These values may be compared with similar values found on analysis of fermented purines from yeast nucleic acid. The values for the decomposable portion of the purines of yeast nucleic acid are slightly lower than the values for pure guanine, but are widely different from the values for xanthine or for hypoxanthine.

Some preliminary work has been done to determine the effect on analytical results of additions of purines to YNA before hydrolysis. Quantitative recoveries of such added purines in the purine N fractions have been achieved. Indications are that quantities of added purine N which are

less than 5 per cent of the total purine N in the sample will not affect the acetic acid and carbon dioxide values enough to be detected. Presence of added hypoxanthine is most easily detected due to the greater relative acid production, as seen in Table IV.

#### DISCUSSION

The specificity of *Clostridium acidiurici* makes possible a quantitative separation of adenine from other commonly occurring purines. A method for the estimation of adenine has been developed in which the non-decomposable purine N is assumed to be adenine N. This assumption is valid in most instances because most biological systems contain only common purines (uric acid, xanthine, guanine, hypoxanthine, and adenine). However, in some cases, large amounts of purines or purine derivatives are present which are precipitable as cuprous complexes and which are non-decomposable by *Clostridium acidiurici*. Therefore, general specificity of the method for adenine cannot be claimed. In spite of this, it is believed that the method has value in purine chemistry. Further work is being done on chemical and microbiological means for the identification of adenine in the non-decomposable fraction of purine mixtures.

Identification of decomposable purines when present singly or in binary mixtures with adenine is made possible by determination of the ratios of ammonia, carbon dioxide, and acetic acid produced. The ammonia production is a measure of the quantity of such purine decomposition. Attempts have been made to estimate amounts and identities of three decomposable purines in mixtures by use of simultaneous equations. But the magnitude of experimental errors shown in Table I is too large for such a method to be successfully employed. The existence of mixtures of decomposable purines can be detected if appreciable quantities of all components are present, but the identification of the individual purines is not yet possible.

Gulland (3) has found the ratio of purine N to pyrimidine N in ribonucleic acid to be 1.86 instead of 2.00 which is predicted by the tetranucleotide formula. From the data given in Table III the purine N to pyrimidine N<sup>3</sup> ratio of the yeast nucleic acid studied may be calculated to be 1.93. However, since any loss of purine N increases the value for pyrimidine N, it is believed that the difference in ratio between 1.93 and 2.00 is well within the experimental error for this determination. The quantities of guanine N and non-decomposable purine N (presumably adenine) are also equal within experimental error. The data thus support the polytetranucleotide formula for yeast nucleic (ribonucleic) acid.

<sup>3</sup> Pyrimidine N calculated as the difference between total N and purine N.

## SUMMARY

1. A microbiological method for the estimation of adenine in the presence of other commonly occurring purines is presented. The method is based on the specificity of *Clostridium acidurici*.

2. Determination and identification of other common purines in binary mixtures with adenine is possible by this procedure.

3. The purine content of a commercial sample of yeast nucleic acid is found to agree very well with the polytetranucleotide formula.

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equipped with the Longworth schlieren scanning device with univalent buffers at an ionic strength of 0.1. Most of the observations were made at a protein concentration of about 1 per cent except in the region of the

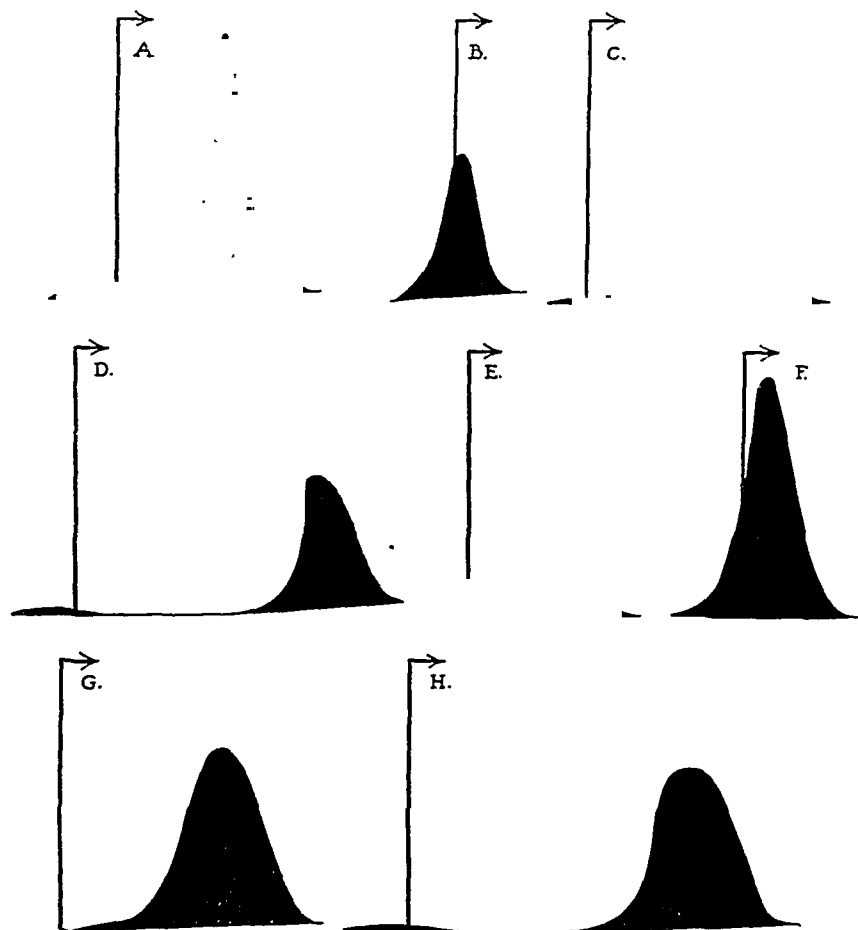


FIG. 1. Descending patterns obtained on electrophoresis of human  $\gamma$ -globulins. The protein concentration was about 1.0 per cent, except for *B* (0.3 per cent) and *D* (0.4 per cent). The photographs were taken at 250 minutes, with the exception of *B*, which was taken at 167 minutes. The II-3 globulin is shown in *A*, veronal buffer, pH 8.55; in *B*, cacodylate, pH 7.25; in *C*, acetate, pH 5.14; and in *D*, acetate, pH 3.43. The II-1,2 globulin is shown in *E*, veronal, pH 8.47; in *F*, cacodylate, pH 6.79; in *G*, acetate, pH 4.15; and in *H*, acetate, pH 3.44.

isoelectric point of the II-3 globulin, where its insolubility necessitated the use of lower concentrations. Some of the patterns which were obtained are shown in Fig. 1.

We were unable to detect the presence of any other serum proteins in these preparations. However, some of the patterns show greater symmetrical spreading than would be expected for electrophoretically homogeneous particles. It is also evident that the patterns obtained at acid pH values are not symmetrical, giving further indication of the inhomogeneity of these  $\gamma$ -globulins. The electrophoretic properties of human  $\gamma$ -globulins have also been recently studied by Alberty (9).

In Fig. 2 the electrophoretic mobilities are shown as a function of pH for both of the  $\gamma$ -globulins as obtained from descending migrations at an ionic strength of 0.1. The apparent isoelectric point of the II-1,2 fraction is at

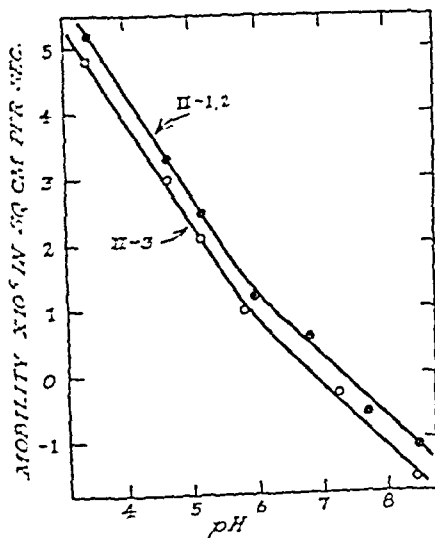


FIG. 2. Electrophoretic mobility as a function of pH for human  $\gamma$ -globulins II-1,2 and II-3. All of the measurements were determined from descending migrations at 1° in univalent buffers at an ionic strength of 0.1. The pH measurements were made with a glass electrode at 25°.

pH 7.3 and of the II-3 fraction at pH 6.85. These values are consistent with the previously recorded differences in the basic amino acid content of these proteins (8).

Human  $\gamma$ -globulins prepared by alcohol fractionation methods have been found to be inhomogeneous in the ultracentrifuge (10). We have made some studies with the preparations used in this investigation and have obtained results that are in agreement with previous observations. The II-1,2 and II-3 preparations contain about 75 per cent of a component with  $S_{20}$  about 6.5 to 7.0 Svedberg units, and 25 per cent of heterogeneous heavier material which sediments over a range of about 9 to 17 S.



units. No material lighter than the principal component was found in either preparation. Observations were made on 1 per cent protein solutions in 0.15 M NaCl at pH 7.0 or in 0.1 M veronal buffer at pH 8.5. The instrument was the electrically driven ultracentrifuge manufactured by the Specialized Instruments Corporation of Belmont, California.

### *Immunological Observations*

*Material and Methods*—Female rabbits were immunized with alum precipitated suspensions of  $\gamma$ -globulin fractions II-1,2 and II-3. The animals received 0.5 to 2.0 mg. of antigen intravenously four times a week for 4 to 6 weeks. For the quantitative precipitin determinations, 0.5 ml of heat-inactivated antisera (56° for 30 minutes) was placed in a 10 ml centrifuge tube. Antigen in 0.5 ml. of 0.15 M NaCl was then added, the fluid was mixed thoroughly, and kept at 4° for 48 hours. The precipitate was centrifuged for 30 minutes at 2000 R.P.M. with a maximum temperature of 7° in the tube. The sediment was drained and then washed with 3.0 ml of chilled 0.15 M NaCl. This was immediately centrifuged and again drained.<sup>1</sup> The protein content of the precipitate was determined by the tyrosine method described by Heidelberger and MacPherson (11). The standard tyrosine curve was constructed from spectrophotometric measurements of  $\gamma$ -globulin II-1,2 in known quantities, as determined by a micro Kjeldahl technique. Values for antigen are expressed as mg. of protein per ml. of fluid. The protein content of the precipitate, in mg., represents the quantity produced by addition of 1 ml. of antiserum to 1 ml. of antigen. For estimation of the  $\gamma$ -globulin content of human serum by the immunological technique, it was necessary to dilute the serum 1:30 to 1:250 before addition of the standardized antiserum. The supernatants always contained an excess of antibody. Duplicate determinations of the protein content of the precipitate were performed.

*Immunological Homogeneity of  $\gamma$ -Globulins*—In Table I the quantitative estimation of the precipitate formed by addition of varying quantities of fractions II-1,2 and II-3 to their homologous antisera is presented. The supernatant fluid was examined for presence of excess of antigen by addition of antiserum and for excess of antibody by addition of antigen. Table I also includes the calculated values for the antibody expected from the quantity of antigen added, with Equations 3 and 6 of Heidelberger and Kendall (5). Equation 3 may be expressed:  $(Ab/An) = (2R - R^2/A(An))$

<sup>1</sup> We have found that a single washing of the precipitate is sufficient to remove the proteins of the supernatant fluid under the conditions specified when the volume of wash fluid is extremely high as compared to the minute quantity of precipitate. In tests where an additional washing was performed, only a negligible color reaction was given by the wash fluid.

where  $Ab$  is antibody and  $An$  is antigen in mg.  $A$  represents the total units of reactive antibody in the system and  $R$  represents the value for  $Ab/An$  at the zone of equivalence, where there is no free antigen or antibody in the system. By plotting the experimentally observed values for  $Ab/An$  against  $An$ , a straight line is obtained for pure antigens. The intercept of the line on the ordinate equals  $2R$  and the slope is represented by  $-R^2/A$ . From these values, it is possible to calculate the amount of antibody which may be obtained by the addition of a known quantity of antigen.

TABLE I  
Quantitative Precipitation Data with Homologous Antigen and Antibody of Human  $\gamma$ -Globulin

Antigen No.	Amount of antigen	Total ppt.	Antibody			Antibody antigen observed	Supernatant	
			Observed	Calculated (Equation 3)	Calculated (Equation 6)		Antigen	Antibody
	mg.	mg.	mg.	mg.	mg.			
II-1,2	0.10	1.0	0.9	0.9	0.9	8.7	4+	—
	0.30	2.5	2.2	2.3	2.3	7.3	4+	—
	0.50	4.1	3.6	3.5	3.4	7.2	4+	—
	0.60	4.4	3.8	3.9	3.8	6.3	4+	—
	0.75	5.7	4.9	4.5	4.4	6.4	4+	—
	0.85	5.7	4.9		4.8	5.7	+	—
	0.95	5.9	4.9		4.9	5.2	—	—
	1.05	5.6	4.6				—	+
II-3	0.10	1.0	0.9	0.9	0.9	9.0	4+	—
	0.20	1.7	1.5	1.6	1.6	7.6	4+	—
	0.30	2.3	2.0	2.2	2.1	6.7	4+	—
	0.40	3.0	2.6	2.6	2.6	6.5	4+	—
	0.50	3.6	3.1	3.0	2.9	6.2	++	—
	0.60	3.8	3.2	3.2	3.2	5.3	+	—
	0.60*	4.0	3.4	3.2	3.2	5.7	+	—
	0.70*	4.4	3.7		3.4	5.2	—	—
	0.80*	4.8	4.0				—	+

\* Serum + antigen stood 96 hours instead of 48 hours in the cold.

Equation 6 is expressed as follows:  $Ab/An = 3R'' - 2[(R'')^3 An/A]^{\frac{1}{2}}$ . The symbols have the same meaning as in the previous equation. Equations 3 and 6 are valid only in the region of antibody excess. It will be observed that the calculated values obtained from these equations are consistent with the observed values for both the II-1,2 and II-3 globulins with the homologous antisera (Table I). The same results were obtained with four different antisera to II-1,2 globulin and three antisera to II-3 globulin.

$\gamma$ -globulin antiserum was estimated for fifteen normal human sera. The values ranged from 1.02 to 2.29 gm. per 100 ml. of sera (13 to 32 per cent of the total serum protein). The mean value was 1.74 gm. or 24 per cent of the total serum protein. The corresponding electrophoretic values for the total  $\gamma$ -globulin in the fifteen sera ranged from 0.30 to 1.25 gm. per

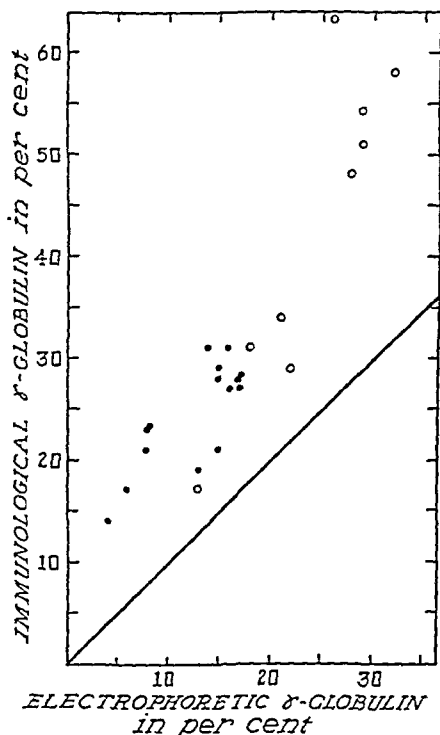


FIG. 4. Electrophoretic estimation of serum  $\gamma$ -globulin as compared with the immunological values. The results are expressed as per cent of the total serum protein. O, abnormal sera, ●, normal sera. The line indicates the theoretical expectation if the two methods give concordant results. The electrophoretic estimations were made at 1.5 per cent protein concentration from descending migrations in veronal buffer at pH 8.4 to 8.6 for 250 minutes. The values are the sum of both  $\gamma_1$  and  $\gamma_2$  components.

100 ml. of serum (4 to 17 per cent of the total serum protein). The mean value was 13 per cent or 0.90 gm. per 100 ml. In Fig. 4, comparative immunological and electrophoretic estimations for  $\gamma$ -globulin, expressed as per cent of total serum protein, are given for fifteen normal sera and for nine abnormal sera. It will be noted that the results for  $\gamma$ -globulin obtained immunologically are considerably higher than the electrophoretically determined values for  $\gamma$ -globulin. In fact, there appears to be

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Although the material which cross-reacts immunologically with  $\gamma$ -globulin is still unknown, certain of its characteristics can be described. It is not albumin since our antisera did not react with human albumin. Therefore, the substance must be present in one of the globulin fractions. In some of the sera, it is present in larger amounts than the electrophoretically estimated  $\gamma$ -globulin, and must constitute a large part of the  $\alpha$ - or  $\beta$ -globulins.

The apparent cross-reaction between  $\gamma$ -globulin antisera and some other component of human serum is not the first instance in which this has been found. Treffers, Moore, and Heidelberger (17) demonstrated that horse  $\beta$ -globulin can react with antisera to horse  $\gamma$ -globulin. Although the human  $\gamma$ -globulins apparently contain most of the known antibodies to disease (13), there is evidence that the T- and  $\gamma$ -globulins of the horse (18) and the cow<sup>2</sup> do not possess all of the antibody activities found in the sera of hyperimmunized animals of these species. Our finding that protein immunologically related to  $\gamma$ -globulin occurs in the  $\alpha$ - or  $\beta$ -globulins suggests that this related protein also may be capable of possessing antibody activity in the human.

#### SUMMARY

1. The human  $\gamma$ -globulin fractions II-1,2 and II-3 possess isoelectric points at pH 7.3 and 6.85, respectively. Although no other serum constituents could be detected electrophoretically in these fractions, both show some evidence of electrical inhomogeneity. Likewise, neither fraction is homogeneous in the ultracentrifuge.

2. Both  $\gamma$ -globulins (II-1,2 and II-3) behave as homogeneous antigens towards rabbit antisera, thus providing additional evidence that the particles of different electrophoretic mobility and size are truly  $\gamma$ -globulins. The two fractions appear to be immunologically equivalent in spite of their known differences in other properties.

3. Antisera to human  $\gamma$ -globulin react with some additional, and as yet unidentified, globulin component of human serum. This yields abnormally high values for immunologically determined  $\gamma$ -globulin when compared with the electrophoretic estimations.

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<sup>2</sup> Unpublished observations by E. L. S.



# THE BIOCHEMISTRY OF THE METABOLIC FECAL PROTEIN NITROGEN

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(Received for publication, June 9, 1948)

In determining the nutritional value of various protein preparations in the infant in terms of nitrogen balance and growth response, it occurred to us that these results would gain in significance if the nature of the fecal proteins excreted under the various regimens were known. Although it is clear from the literature that the amount and composition of the fecal fat are to a large extent independent of the dietary fat (1), we were unable to find any clear evidence on the effect of dietary proteins on the composition of the fecal proteins (2). The attempt to assess the nature of this relationship raised the problem (a) of finding a suitable method for the quantitative determination of fecal proteins and (b) of isolating suitable quantities of these proteins for amino acid analysis. Heretofore only qualitative or indirect quantitative (3) procedures have been employed for the estimation of fecal proteins.

After a number of preliminary experiments we found that the fecal protein could be determined quantitatively by a micro-Kjeldahl analysis of the proteins precipitated isoelectrically at pH 6.0 from an alkaline ethanol extract of an aqueous homogenate of the feces. The analytical adequacy of this technique was further demonstrated by recovery tests and by its failure to measure proteins of the intestinal flora. Application of the method to the stools of ten infants on various diets for periods ranging from 1 to 16 weeks revealed that an average of 22.4 per cent of the fecal N arises from the protein moiety and that fluctuations from this mean value are spontaneous and not related to the diet.

Analyses of fecal proteins isolated by a modification of the analytical procedure from the stools of these infants disclosed that the amino acid composition of these products was unaffected by the use of whole milk proteins, casein-, tryptophan-, isoleucine-, or lysine-deficient preparations as the nitrogen component of the diet. This observation, the absence of bacterial bodies from the protein fraction, and the uniformity of amino acid pattern of the different fecal protein specimens suggest that this stool fraction is not derived from residues of the dietary N or from the bodies of the intestinal bacteria, but from definite intestinal secretions.

## EXPERIMENTAL

Exploratory tests disclosed that the fecal protein could be isoelectrically precipitated from the alkaline alcoholic extracts by adjustment of the pH to  $6.0 \pm 0.1$  with HCl,  $H_2SO_4$ , or trichloroacetic acid. The isoelectric point of this protein is to be compared with that of casein, 4.6, and lactalbumin, 4.5 to 5.5 (4). The protein can also be precipitated by half saturation of the extract at pH 7.0 with ammonium sulfate. The negative Exton, biuret, and trichloroacetic acid tests, given by filtrates from the isoelectric or salt precipitation of the protein, indicated that either of these two procedures could be employed in the quantitative measurement of the fecal protein N.

*Determination of Fecal Protein N*—24 hour stools were collected in wide mouth preserving jars containing 40 cc. of 70 per cent ethyl alcohol. Prior to removing samples for analysis the volume of the mixture was adjusted to 200 cc. with water and homogenized with a mechanical stirrer. (5 day pools were collected in 200 cc. of 70 per cent ethanol and made to 1 liter with water before analysis.) To duplicate 10 cc. aliquots of the homogenate, which were transferred into 40 cc. graduated centrifuge tubes by means of a wide tipped Mohr pipette, were added 1 cc. of 10 per cent NaOH and 95 per cent ethanol to the 20 cc. mark. These specimens were then stirred mechanically for 5 minutes with an air-driven stirrer and centrifuged for 10 minutes at 3000 R.P.M. A clear supernatant fluid was usually obtained; if it remained cloudy it was clarified by filtering through fluted Whatman paper No. 12. To 10 cc. aliquots of the clear fluid in 15 cc. centrifuge tubes were added 5 drops of brom-cresol purple indicator (0.05 per cent alcoholic solution) and the pH adjusted to 6.0 with 40 per cent trichloroacetic acid (about 0.7 cc. is required). The reaction mixture was stored in the refrigerator for 30 minutes and then centrifuged. The supernatant fluid was discarded and the precipitated protein was washed by resuspension in 5 cc. of cold 5 per cent trichloroacetic acid, centrifugation and decantation of the supernatant fluid. The washed protein precipitates were then transferred quantitatively to 100 cc. Kjeldahl flasks with the aid of a minimum amount of water and the nitrogen content was determined by the micro-Kjeldahl procedure of Meeker and Wagner (5). The analytical adequacy of the single extraction was demonstrated by the absence of protein in a second alkaline alcohol extract of the first extraction residues. The total N of the stool suspension was determined by direct micro-Kjeldahl analysis of 10 cc. aliquots of the homogenate.

*Isolation and Analysis of Fecal Protein*—In order to avoid contamination of the fecal protein by residues of a previous dietary regimen, the various diets were fed for 2 or more successive weeks and the fecal protein isolated from the feces collected during the last week of the diet period.



Aliquots of the 5 day pools of two or three infants, sufficient to make a total volume of 2 liters, were employed for each preparation. These samples were combined in a 5 liter round bottom flask and treated as described above with a proportionate increase of all the reagents. The final reaction mixture was stored overnight in a refrigerator at 4°. The precipitated protein was collected by decantation of the supernatant liquid, purified by electro dialysis, collected by centrifugation, and granulated by successive suspension in 50, 75, and 95 per cent acetone. This product was dried in a stream of compressed air and then in a 37° oven for 24 hours. The total N, moisture, and ash contents of each sample were determined.

Subsequently acid and alkaline hydrolysates of these proteins were prepared. The alkaline hydrolysate was employed for the determination of tryptophan by the procedure described by Albanese and Frankston (6). The other amino acids were determined in hydrolysates prepared by refluxing 1.5 gm. of the proteins with 10 cc. of 6 N HCl for 24 hours. The total N content of the hydrolysate was determined directly by micro-Kjeldahl analysis; then the excess of acid was removed by concentration *in vacuo* and the humin was separated by filtration. The volume of this filtrate was adjusted to 50 cc. and appropriate samples were removed for the various amino acid analyses.

*Metabolism Experiments*—The fecal specimens employed in this study were collected from normal healthy male infants who were given the various diets in five feedings daily at the rate of approximately 100 calories per kilo of body weight and 500 mg. of ascorbic acid together with 15 drops of oleum percomorphum daily. The diet periods were of 7 days duration and consecutive, but excreta collections were omitted on week-ends to avoid complications which might arise from the continued use of restraints. The subjects were immobilized by abdominal restraints which were also designed to hold the urinary adapters in place. 24 hour urine specimens were collected in bottles containing 10 cc. of 15 per cent (by volume) HCl and 1 cc. of 10 per cent alcoholic thymol.

The feces were collected in 19 cm. porcelain evaporating dishes which were held in place by a properly shaped excavation in the mattress and the daily stools were accumulated under refrigeration for each period in jars containing 200 cc. of 70 per cent ethanol. The subjects were weighed daily during the course of the investigation.

The synthetic diets fed in this experiment contained approximately 100 calories per 100 gm., the percentage caloric distribution in all instances being as follows: protein 14, fat 36, carbohydrate 50. The composition of the synthetic diets is shown in Table I. The evaporated milk formula employed in the control experiments (Table IV) had the following composition: evaporated milk 40 cc. (55.3 calories), corn syrup 6 cc. (17.7 calories),

water 54 cc. This supplied approximately 73 calories per 100 cc. and the nitrogen content of each batch was controlled by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24 hour urine collections, analysis of the pooled feces for each period, and from computation of the daily nitrogen intake

TABLE I  
*Composition of Diets*

All diets were fed at the rate of 100 calories and 3.5 gm. of protein ( $N \times 6.25$ ) per kilo of body weight.

	Diets				
	Casein	Gluten	TH	CTH	BHb
	gm.	gm.	gm.	gm.	gm.
Casein (Sheffield).....	3.5	0	0	0	0
Gluten (Interchemical).....	0	3.5	0	0	0
Acid-hydrolyzed casein*.....	0	0	3.5	3.4	0
"    beef hemoglobin.....	0	0	0	0	3.4
L-Tryptophan.....	0	0	0	0.06	0.06
L-Cystine.....	0	0	0	0.04	0.04
Brewers' yeast.....	1.0	1.0	1.0	1.0	1.0
Olive oil.....	4.0	4.0	4.0	4.0	4.0
Dextri-Maltose No. 2†.....	9.6	9.6	9.6	9.6	9.6
Arrowroot starch.....	2.3	2.3	2.3	2.3	2.3
Salt mixture‡.....	1.6	1.6	1.6	1.6	1.6
Water.....	78.0	78.0	78.0	78.0	78.0
Total.....	100.0	100.0	100.0	100.0	100.0
Estimated content of deficient amino acid.....		mg. 52	mg. 6	mg. 59	mg. 36

\*  $N \times 6.25 =$  gm. of protein.

† Kindly supplied by Mead Johnson and Company.

‡ The salt mixture employed had the following composition (measured in gm.):  $\text{FeSO}_4$  0.9,  $\text{NaCl}$  6, calcium gluconate 48,  $\text{Ca(OH)}_2$  12,  $\text{KH}_2\text{PO}_4$  7,  $\text{KCl}$  6,  $\text{MgO}$  0.1.

based on food consumption records and the known nitrogen content of the diets.

### Results

The efficacy of the analytical procedure described previously was evaluated by recovery tests in which various biological products were added to the fecal homogenates. It is clear from these experiments (Table II) that the quantitative isoelectric precipitation of fecal protein is not influenced by the presence of the proteins of cow's milk or human plasma, non-protein nitrogenous substances, or the nitrogen of bacterial bodies.

soluble in 10 per cent NaOH, 95 per cent formic acid, and 40 per cent urea solution, and contained no carbohydrate component. Moreover, all purified specimens of the isolated protein showed no proteolytic activity towards

TABLE III

*Fecal Protein Nitrogen Output of Male Infants Fed Evaporated Milk*

All the results are expressed as daily averages.

Subject	Age	Body weight	Nitrogen retained	Total fecal protein	Fecal protein N of total N	Fecal protein N of retained N	Period I				Period II			
							Fecal protein N of retained N	Nitrogen retained	Total fecal protein	Fecal protein N of total N	Fecal protein N of retained N	per cent	per cent	per cent
De.	3	4.6	790	400	72	18.0	9.1	870	350	61	17.5	7.0	14.2	6.9
Ja.	4	4.0	520	400	69	17.3	13.3	690	470	98	21.1	7.0	24.8	10.4
Ma.	8	8.5	1000	560	180	32.4	18.0	840	640	208	32.2	24.8	10.4	5.6
Sh.	2	3.3	1050	450	111	24.8	10.6	840	400	87	21.9	10.4	5.6	6.9
Od.	9	8.5	1030	440	70	15.7	6.8	1380	280	77	21.6	6.9	5.6	6.9
Sm.	4	3.6	860	320	75	23.2	8.7	950	300	61	20.1	6.9	5.6	6.9

TABLE IV

*Effect of Various Diets on Fecal Protein Nitrogen Output of Male Infant*

All the results are expressed as daily averages.

Subject	Age	Body weight	Diet	Nitrogen retained	Total fecal protein	Fecal protein N of total N	per cent
Sa.	7.5	6.3	Casein	1090	143	23.5	3.1
Go.	14.0	6.8	"	530	151	44.5	12.6
Ge.	25.7	9.6	"	2360	204	28.8	2.5
Sa.	9.5	7.1	CTH	1890	160	19.7	1.6
Go.	16.0	7.3	"	470	230	17.1	8.3
Ge.	27.7	9.9	"	1150	304	36.7	9.6
Go.	16.5	7.4	TH	260	412	15.8	25.0
Ge.	28.2	10.0	"	320	304	12.7	11.9
Od.	11.0	8.2	BHP	550	314	20.7	11.8
Ja.	7.2	6.0	"	420	416	23.6	23.0
Ca.	3.0	6.5	"	320	292	23.3	21.0
De.	7.0	5.6	Gluten	560	340	38.8	23.2
Ja.	4.2	4.8	"	540	418	35.3	27.4

casein (8) and no arginase activity towards D-arginine (9). The results of amino acid analysis of the various specimens of the protein are collected in Table V. On the basis of the agreement of the values for ten of the amino

acids and previously mentioned characteristics, it would seem reasonable to assume that the different samples represent a single protein. The possibility of a dissimilarity of pattern for the remaining amino acids, of course, exists, but this possibility is contraindicated by the uniformity of the organic acid: amino N ratios found for the different samples. The lysine content of the protein, 12.2 per cent, is uncommonly high (21). Finally, the presence of tryptophan, isoleucine, and lysine in the fecal protein isolated from the stools of infants fed hydrolyzates deficient in these amino

TABLE V

Analysts of Fecal Protein Isolated from Feces of Infants Fed Various Diets  
The amino acid values are given as per cent amino acid N of total N.

Diet																																																																																	
Eradicated milk	Casein	CTH	TH	BHB	Gluten	Moisture content, %						Ash content, %					Corrected N content, %					Ratio, organic acid (10)* to amino N†					Amino N (11)					Threonine (12)					Arginine (13)					Histidine (14)					Lysine (15)					Cysteine (16)					Methionine (17)					Isoleucine (18)					Phenylalanine (19)					Tyrosine (20)					Tryptophan (6)				
						7.63	7.27	7.55	8.88	7.27	3.85	0.11	0.17	0.17	14.59	14.67	0.67	0.69	82.5	81.6	5.7	5.8	12.6	12.7	5.2	5.7	16.2	16.1	1.3	1.4	1.9	1.8	4.3	3.1	2.8	2.0	3.4	3.3	7.63	7.27	7.55	8.88	7.27	3.85	0.11	0.17	0.17	14.59	14.67	0.67	0.69	82.5	81.6	5.7	5.8	12.6	12.7	5.2	5.7	16.2	16.1	1.3	1.4	1.9	1.8	4.3	3.1	2.8	2.0	3.4	3.3										

\* The figures in parentheses represent bibliographic references.

† This ratio was obtained by dividing the acid titer (expressed as cc. of 0.1 N HCl) by mg. of amino N of the sample.

acids should dispel any further doubts regarding the dietary origin of the fecal protein.

Comments

In 1892 Voit (21) found that the excretion of substances from an isolated loop of the intestine of dogs produced a mass similar in constitution and nitrogen content to that produced in the normal intestine of the same animal through which meat and fat were passing. He therefore concluded that the feces are derived principally from the substances excreted through

the wall of the intestine and that the nitrogen so excreted is as much to be considered a product of protein metabolism as is the nitrogen of urea. He expressed regret at that time that very little was known regarding the chemistry of these nitrogenous compounds excreted into the intestine.

Subsequently Schneider (22) was able to show clearly that two distinct fractions of metabolic nitrogen exist in the feces. One fraction is constant for each animal but varies among different animals roughly in proportion to size. This is the truly excretory component and would be represented by the fecal material produced during fast. The other fraction varies in proportion to the intake of dry matter. This component is a true digestive waste. In Mitchell's opinion (23) the excretory fraction of the metabolic nitrogen is so small that on amounts of food permitting maintenance of weight or growth its effect on the ratio of total metabolic nitrogen to dry matter consumed is negligible. In a later publication Schneider (24) reported that "contrary to results on rats and pigs, the results on human subjects indicate that all of the metabolic nitrogen of human feces is proportional to food intake." This viewpoint is in contradiction to the conclusions of Thomas (25) and Martin and Robison (26), who considered all of the metabolic nitrogen of human feces to be constant. Obviously, the existence and nature of the metabolic fecal nitrogen are a matter of considerable significance in the determination of the biological value of dietary proteins. It would appear from the observation reported here that in the infant one of the components of the metabolic fecal nitrogen is a protein. The evidence presented indicates that the amount and composition of this excreted protein do not seem to be influenced by the quantity or quality of the diet.

These findings strongly suggest that the protein which we have described is an endogenous excretory product which might be appropriately named *fecamin*. Attention is called to the fact, however, that the occurrence of proteins in the stools of infants and adults has been previously reported by other investigators who generally attributed the origin of these proteins to exogenous sources, e.g. food residues, end-products of internal hemorrhage, pathological exudates, and bacterial bodies (3, 27). However, since no attempt was made to isolate or characterize the fecal proteins in these previous studies, the erroneous conclusions drawn regarding the origin of the proteins are easily understandable. Moreover, the presence of dietary protein residues in the isolated protein fraction could not be excluded beyond a doubt in the present study except by the substitution of protein hydrolyzates for the protein moiety of the diet. It is obvious that when poorly assimilated proteins are present in the diet or conditions of poor assimilation prevail in the organism, the differentiation of the metabolic and waste proteins in the feces may prove a difficult, if not impossible task.

The isolation and proof of identity of the metabolic fecal protein in the adult could probably be achieved under conditions of starvation or artificial alimentation. Apparently under conditions of prolonged fasting such as reported by Benedict (28) and Paton and Stockman (29) this excretion did not occur, or was so reduced in quantity as to escape measurement. It is clear from the foregoing that the nitrogen lost in the feces in the form of fecal protein constitutes a loss of anabolic N. Although this loss constitutes an average of only 11.9 per cent of the retained N, the high content of essential amino acids of this fraction greatly augments its significance. This is particularly true when poor quality or deficient diets are fed. Furthermore, since the formation of the proteins involves a considerable biosynthetic effort on the part of the organism, the loss must affect the nitrogen economy of the organism far more than the N content of the protein would indicate. These considerations would seem to make a study of the factors concerned in the formation of the fecal protein and of its physiological functions worth while.

#### SUMMARY

In infants fed experimental diets it has been found that an average of 22.4 per cent of the fecal N arises from the presence of a protein which failed to reflect in its amino acid composition and other properties the dietary changes imposed on the organism from which it was derived. It is believed that this protein constitutes a moiety of the excretory or metabolic fecal N and its implications on the calculations of the biological value of dietary proteins are discussed.

We are gratefully indebted to Dr. Selma E. Snyderman for the medical care of the subjects.

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# THE AMMONIA AND GLUTAMINE CONTENT OF THE BRAIN

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(Received for publication, July 29, 1948)

The values given in the literature for the ammonia content of the brain show wide variation (1-3). Most of these values are of doubtful reliability, since the methods of estimation have not generally taken into account the presence of labile acid amides such as glutamine, which are now known to occur in the brain and which easily liberate ammonia on treatment with alkalis under the conditions commonly used for the estimation of ammonia (4, 5). Interest in the brain ammonia level centers around the fact that ammonia is a powerful cerebral irritant; the administration of ammonium salts causes convulsions and it has long been thought that ammonia may play a part in the precipitation of epileptic seizures (3-6). It would appear that the brain ammonia level is closely related to the glutamic acid-glutaminase system, which can remove ammonia by combination with glutamic acid to form glutamine (7). The report that glutamic acid inhibits ammonium chloride convulsions (8) may be significant in this connection, since it is known to reduce the incidence of attacks of petit mal in epileptics (9).

The present paper reports an attempt to obtain reliable values for the ammonia content of rat brain with a method by which the free ammonia can be distinguished from the labile acid amide ammonia. At the same time a study has been made of the effect on the brain ammonia level of convulsant drugs and other factors which might affect it.

## EXPERIMENTAL

*Ammonia Estimation*—Ammonia was estimated by Conway's method (10) of microdiffusion analysis. The acid used was 0.0004 N HCl containing Tashiro's indicator, prepared as described by him (11). The baryta solution (0.0015 N) was delivered from an Agla micrometer syringe (12), which was capable of delivering as little as 0.0001 ml. or considerably less than the error inherent in detecting the indicator change at these dilutions. The stock baryta solution was kept in a rubber-capped bottle of the type used for keeping sterile solutions for injection, which gave a convenient method of refilling the syringe without contamination by CO<sub>2</sub> in the air; the solution was restandardized each day. Preliminary tests with pure crystalline glutamine showed that the rate of liberation of ammonia by



hydrolysis was 0.001 mg. of ammonia per mg. of glutamine per hour in contact with 50 per cent-saturated  $K_2CO_3$  at  $20^\circ$  under the experimental conditions of the Conway method. The glutamine in the brain filtrate was determined separately and a correction was calculated on this basis for the brain ammonia figures. The magnitude of the correction was kept low by using a diffusion time of 1 hour. Glutamine is relatively stable at neutral pH at  $0^\circ$ , but it was found to hydrolyze at an appreciable rate, 0.00042 mg. of  $NH_3$  per mg. of glutamine per hour being liberated at a room temperature of  $18^\circ$  in contact with 10 per cent trichloroacetic acid, which was used at approximately this strength in the brain filtrates; the error due to this cause was reduced to a negligible figure by carrying out the ammonia estimations without delay and keeping the solution at  $0^\circ$  with ice. An estimation on a standard solution of ammonium chloride was included in each series.

*Glutamine Estimation*—Glutamine was estimated by the method of Harris (13), which depends on estimating the ammonia liberated on hydrolysis under standard conditions with 10 per cent trichloroacetic acid at  $70^\circ$  for 75 minutes. Preliminary tests confirmed the observation of Harris that under these conditions a solution containing 50 mg. per cent of pure glutamine was hydrolyzed approximately 100 per cent; under the same conditions asparagine was hydrolyzed 11 per cent, acetamide 69 per cent, and nicotinamide 4 per cent. There was no appreciable liberation of ammonia from D-arginine, guanidine, guanine, adenine, creatine, glutamic acid, or metrazol, but urea liberated 0.002 mg. of  $NH_3$  per mg. under these conditions. Although crystalline glutamine has been isolated from horse brain (14), it was not known to what extent other labile acid amides might be present in the brain. To obtain further evidence on this point, measurements were made of the rates of hydrolysis of the labile acid amides of the brain filtrates and the figures were compared with the rates of hydrolysis of pure glutamine solutions under the same conditions. Four experiments in which a comparison was made of the amount of ammonia liberated from the brain filtrate and from an equivalent amount of pure glutamine on hydrolysis by 50 per cent-saturated  $K_2CO_3$  at  $20^\circ$  are summarized in Table I. Hydrolysis curves for brain filtrates under acid conditions, carried out as described by Harris (13), also showed good agreement with the curves for pure glutamine, the specific reaction rate  $k$  being  $7.7 \times 10^{-4}$  in each case. This gave evidence that the labile acid amide of the brain is mainly, if not wholly, glutamine, and the results are therefore expressed in terms of this substance.

*Experimental Animals*—In most of the work, young Wistar albino rats weighing 25 to 40 gm. were used. They were killed by being dropped into liquid air, which froze them solid in a few seconds and produced a rapid

1.05 mg. per cent of ammonia with a mean of 0.93 mg. per cent, but by reducing the interval between decapitation and fixing in liquid air lower values were obtained (Table V). With an interval of 1 second between decapitation and freezing in liquid air a mean value of 0.47 mg. per cent of ammonia was obtained and this was adopted as a standard procedure in the experiments with adult rats, which were always killed by decapitation.

TABLE V

*Ammonia Content of Rat Brain (a) after Anoxia in Vivo and (b) at Various Times after Decapitation*

In series (a) young rats of 30 to 40 gm. were rendered anoxic by keeping them for 1 minute in nitrogen containing 5 per cent CO<sub>2</sub>; they were killed by decapitation and the heads were frozen after 1 second. The brain ammonia figures should be compared with the mean of 0.47 mg. per cent for normal, decapitated rats. In series (b) the heads of rats of 200 to 300 gm. were transferred to liquid air at varying times after decapitation. The heads were kept at 37°.

Rat No.	Anoxia			Change after decapitation		
	Brain ammonia	Blood ammonia	Remarks	Rat No.	Time after decapitation	Brain ammonia
	mg. per cent	mg. per cent			sec.	mg. per cent
1	0.64	0.46	Strong convulsions	6	$\frac{3}{4}$	0.29
2	0.96	0.80	Slight tremors	7	1	0.33
3	0.74		Brief convulsion	8	1½	0.62
4	0.54	0.65	Preconvulsive	9	2	0.86
5	1.18	0.16	"	10	3	0.77
				11	4	0.76
				12	4	1.05
				13	5	0.92
				14	10	0.94
				15	15	0.92
				16	30	1.01
Mean..	0.81	0.52				

*Effect of Anoxia*—Rats kept for 1 minute in a desiccator containing nitrogen with 5 per cent CO<sub>2</sub> showed a marked increase in the brain ammonia content (Table V); this was observed in animals which had convulsions and also in rats which had not had convulsions but were in the preconvulsive state. It is known that anoxia causes an increase in the blood ammonia level (18) and this may account for a part of the ammonia found in the brain; but it is likely that the blood ammonia comes from the brain and other tissues rather than that the tissue ammonia comes from the blood.

*Effect of Narcosis*—Rats anesthetized for 30 minutes or longer with nembutal showed a marked fall in the brain ammonia content, the mean level for young rats killed by immersion in liquid air being 0.06 mg. per cent of ammonia. The figures, which are listed in Table VI, give evidence that the brain ammonia depends on the duration of the narcosis, for two animals anesthetized for shorter periods of 10 and 17 minutes showed no significant change from the normal. This may be the explanation of the

TABLE VI

*Effect of Nembutal Narcosis on Ammonia and Glutamine Content of Rat Brain*

The brain ammonia figures should be compared with the mean of 0.47 mg. per cent for normal, decapitated rats and 0.28 mg. per cent for normal rats killed by immersion in liquid air.

Rat No.	Weight of rat	Method of killing	Duration of narcosis	Brain ammonia	Brain glutamine
	gm.		min.	mg. per cent	mg. per cent
1	200-300	Decapitation	30	0.15	86
2	200-300	"	30	0.26	84
3	200-300	"	55	0.13	79
4	200-300	"	65	0.23	83
Mean.....				0.19	83
5	35-40	Liquid air	30	0.05	80
6	35-40	" "	45	0.06	
7	35-40	" "	70	0.15	
8	35-40	" "	80	0.02	
9	35-40	" "	90	0.01	
Mean.....				0.06	
10	35-40	Liquid air	10	0.29	72
11	35-40	" "	17	0.23	95

negative findings of Bülow and Holmes (1), who found that narcosis had no effect on the brain ammonia level.

*Effect of Electrical Stimulation*—Electrical stimulation of the brain was carried out with stainless steel electrodes of 0.25 sq. cm. area, which were applied to the scalp 0.5 cm. posterior to the eyes. Contact was obtained by cutting the fur in this region with sharp scissors and applying electrode jelly. The current used was 50 cycles a.c. at 40 volts. Stimulation for 1 to 3 seconds produced a satisfactory convulsion after the usual latent period of about 10 seconds. The procedure was similar to that used in the "electroshock" treatment of psychiatric patients. In the experiments

listed in Table VII the rats were sacrificed while undergoing strong convulsions and the figures showed a brain ammonia content above the normal.

*Brain Ammonia in Preconvulsive State*—The experiments on stimulation by anoxia showed a rise in the brain ammonia level in the preconvulsive state, before the onset of convulsions, which suggested that the rise in brain ammonia might be associated with the increased cerebral irritability rather than with the actual convulsions. The further experiments given in Table VIII confirmed this impression, showing a clear rise in the brain

TABLE VII

*Ammonia Content of Rat Brain during Convulsions Induced (a) by Electrical Stimulation and (b) by Injection of Ammonium Chloride*

The rats were killed by immersion in liquid air or decapitation on the first strong convulsion. The heads of decapitated rats were transferred to liquid air in 1 second. The ammonia content of controls killed by liquid air was 0.27 and killed by decapitation 0.47 mg. per cent. Ammonium chloride convulsions were induced by intra-peritoneal injection of 1.2 ml. of 20 per cent  $\text{NH}_4\text{Cl}$ .

Convulsions	Rat No.	Weight of rat	Method of killing	Duration of shock	Brain ammonia	Brain glutamine	Blood ammonia
		gm.		sec.	mg. per cent	mg. per cent	mg. per cent
Electric shock	1	35-40	Liquid air	2	0.40	86	
	2	35-40	" "	3	0.49	76	
	3	200-300	Decapitation	1	0.58	79	
	4	200-300	"	1	0.75	82	
	5	200-300	"	3	0.78		1.42
	6	200-300	"	3	0.94		0.69
Mean, decapitated rats only .....					0.76		
Ammonium chloride	7	200-300	Decapitation		8.0	70	8.3
	8	200-300	"		9.5	93	9.8
	9	200-300	"		9.2	101	10.0

ammonia level in animals sacrificed 1 second after electrical stimulation and therefore several seconds before the onset of convulsions. Estimations of blood ammonia carried out on blood from the carotid arteries of rats killed by decapitation showed a rise in the blood ammonia during electrically induced convulsions; this was to be expected, since it is known that ammonia is liberated in the muscles and enters the blood during severe muscular activity (18). Determinations of blood ammonia on rats in the preconvulsive state gave values which were generally in the normal range, but in a few cases the figures were high; this might be due to the diffusion into the blood of ammonia liberated in the muscles through the tonic mus-

cular spasm produced by the electrical stimulation. The wide disparity between the ammonia levels in the blood and the brain observed in these and in other experiments gave evidence that the brain ammonia was formed in the brain itself and could not be attributed as a rule to ammonia entering the brain from the blood.

Since the brain ammonia content was raised in the preconvulsive state, it appeared of interest to find out how far the cerebral irritability in this

TABLE VIII

*Ammonia Content of Rat Blood and Brain in Preconvulsive State after (a) Picrotoxin Administration and (b) Electrical Stimulation*

The rats were killed before the onset of convulsions. Other conditions as in Table VII; picrotoxin dose, 1 ml. of 3 per cent solution.

	Rat No.	Weight of rat	Method of killing	Duration of shock	Brain ammonia	Blood ammonia
		gm.		sec.	mg. per cent	mg. per cent
Picrotoxin	1	200-300	Decapitation		0.65	0.11
	2	200-300	"		0.49	0.42
Electric shock	3	35-40	Liquid air	1	0.40	
	4	35-40	" "	1	0.73	
	5	35-40	" "	1	0.44	
	6	35-40	" "	1	0.41	
Mean, rats killed by liquid air only.....					0.49	
	7	200-300	Decapitation	2	0.71	0.38
	8	200-300	"	3	0.83	0.13
	9	200-300	"	4	1.04	0.22
	10	200-300	"	3	1.10	0.93
	11	200-300	"	2	0.92	0.32
	12	200-300	"	3	0.92	0.70
	13	200-300	"	2	1.10	0.17
	14	200-300	"	2	0.93	0.20
Mean.....					0.94	0.38

state could be attributed to the toxic action of the free ammonia. It was found that, when ammonium chloride was administered by intraperitoneal injection, convulsions occurred at the time when the brain ammonia level was approximately 9 mg. per cent, or about 10 times the level in the preconvulsive state (Table VII). The brain glutamine content appeared to be raised after ammonium chloride administration, but accurate figures could not be obtained in this series, owing to the large amount of ammonia present. In contrast to the figures on ammonia, the brain

tamine values showed little variation with the various experimental procedures which were tried.

#### DISCUSSION

It was found that the ammonia content of the rat brain, analyzed after rapid fixation by freezing in liquid air, was not constant but depended on the state of activity of the brain at the time of fixation. Values obtained on anesthetized animals were significantly lower than those for normal controls, while much higher values were obtained in animals taken during convulsions produced by convulsant drugs or by other methods of stimulation. The brain ammonia content during electrically induced convulsions was more than eight times that found in anesthetized animals. Unless it is believed that these changes occurred during the brief period of freezing with liquid air, it must be concluded that they represent changes occurring *in vivo*. This view is supported by the work of Tashiro (11), who showed that free ammonia is liberated by the isolated frog nerve and that the amount liberated is increased on stimulation. The observations on the frog nerve were confirmed by Winterstein and Hirshberg (19).

The present work showed that the brain contains some source which can liberate up to about 1 mg. per cent of ammonia within 5 seconds upon stimulation. The source of this ammonia is not yet clear, but the brain glutamine content showed little variation, which made it unlikely that glutamine was the source. It might come from the deamination of nucleosides; this has generally been regarded as a relatively slow process (20), but recent work has shown that the simultaneous dephosphorylation and deamination of adenosine diphosphate can proceed with considerable rapidity in muscle preparations (21). It is noteworthy that the liberation of ammonia did not run parallel to the formation of lactic acid in the brain by glycolysis. The brain ammonia and lactic acid are both increased by convulsants and decreased by anesthetics, but there was no liberation of ammonia corresponding to the rise in lactic acid in the brain in emotional excitement.<sup>1</sup> The rapid liberation of ammonia after decapitation came to an end in a few seconds, while the lactic acid formation by postmortem glycolysis continues for a much longer time. Again, the fall in the ammonia content of the brain in anesthesia appeared to follow some time after the decrease in the lactic acid level.

The rise in the brain ammonia content in electrical convulsions was not due to the convulsions themselves, for it could be shown that the liberation of ammonia in the brain occurred in the preconvulsive stage of cerebral irritability before the start of the convulsions. This raised the question of whether the convulsions could be attributed directly to the toxic action of the ammonia. This suggestion was previously made for epileptic con-

vulsions by Riebeling (3) and Brühl (22), who reported that the ammonia content of the cerebrospinal fluid was increased in epileptics after seizures. Richter, Dawson, and Rees<sup>2</sup> were unable to confirm that observation but that did not disprove their hypothesis, for it is not likely that the cerebrospinal fluid would give an accurate reflection of the rapid changes occurring in the brain. The view that epileptic seizures may be attributable to the toxic action of ammonia is attractive, as it can explain a number of the experimentally observed facts; these include (a) the precipitation of seizures by anoxia, which has been shown to liberate ammonia in the brain, (b) the autocatalytic spread of the discharge, as ammonia liberated at one point stimulates the neighboring regions of the brain, and (c) the specific effect of glutamic acid in inhibiting certain types of seizures, in which the rate of detoxication of ammonia might be the limiting factor.

Further experiments designed to test this hypothesis showed that the ammonia liberated in the rat brain on stimulation reached about a tenth of the concentration required to produce convulsions in a normal animal. The figure for the convulsant level was obtained by injecting ammonium chloride and determining the brain ammonia content after the convulsions had started. Under these conditions the ammonia would be fairly evenly distributed throughout the vascular bed and substance of the brain, while it might be expected that the ammonia liberated on stimulation would be formed mainly in the metabolically active regions, where local concentrations higher than the average for the whole brain would be found. The apparent discrepancy between the ammonia level after stimulation and the convulsant level may therefore be less in fact than these experiments would appear to indicate. The present investigation gives evidence that ammonia is liberated in the brain on stimulation and shows that the brain ammonia concentration can approach the toxic range; it suggests that in conditions such as epilepsy, in which the brain is abnormally irritable, the toxic action of ammonia may play a significant part.

#### SUMMARY

Values are reported for the ammonia and glutamine content of the rat brain. The normal ammonia content, determined by a method which avoided the error due to the decomposition of labile acid amides, was 0.28 mg. per cent and the glutamine content was 79 mg. per cent. A study of the factors affecting the brain ammonia level showed that it was decreased by prolonged nembutal narcosis and markedly increased by direct stimulation of the brain or by procedures which increase cerebral irritability. The ammonia level was increased to 0.47 mg. per cent by picrotoxin ad-

<sup>2</sup> Richter, D., Dawson, R. M. C., and Rees, L., *J. Ment. Sc.*, in press.

ministration and 0.81 mg. per cent by anoxia. Electrical stimulation caused a rapid increase in the ammonia level in 1 to 2 seconds and it was also raised by the stimulus of decapitation. The brain ammonia was not affected by emotional excitement and the glutamine content was not significantly affected by any of the factors which were tested. Injection of ammonium chloride in the rat caused convulsions when the brain ammonia level had risen to 9 mg. per cent. The significance of these data in relation to the mechanism of epileptic seizures is discussed.

The authors thank the Medical Research Council for a personal grant to one of us (R. M. C. D.) and for a grant for expenses; they thank the Rockefeller Foundation and the Royal Society for grants for equipment.

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# BIOTIN AND FAT-SOLUBLE MATERIALS WITH BIOTIN ACTIVITY IN THE NUTRITION OF MOSQUITO LARVAE

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(Received for publication, July 6, 1948)

Recent work has shown that certain microorganisms can under appropriate conditions dispense with biotin if they are supplied with oleic acid or other related materials (1-5). Moreover, an oil from hydrolyzed plasma, as well as a partially purified fraction obtained from it, has been found to have biotin-like activity when injected into young chicks fed a diet high in egg white (2, 6). Oleic acid did not have such activity (6). It seemed of interest to investigate the relationship between biotin and fat-soluble biotin-active substances in the nutrition of an organism entirely different from either bacteria or vertebrates. The yellow fever mosquito *Aedes aegypti* was chosen for this purpose for several reasons. It is among the few insects concerning the nutritional requirements of which considerable information is already available (7). It can easily be maintained in large numbers under laboratory conditions. Its larvae develop rapidly so that definite effects on growth can be discerned within a period of 1 or 2 weeks. The larvae are adapted to life in a liquid medium and can utilize both solid foods and nutrients in solution (8). Finally, *Aedes aegypti* larvae can readily be reared free from microorganisms (9).

This last point cannot be too strongly emphasized. It has become increasingly apparent that some effects of diet may be produced only secondarily via an effect on the synthetic activities of the intestinal bacteria (10-13). Since in nature mosquito larvae feed on microorganisms, nothing whatever can be discovered about their nutritional requirements if living microorganisms are present. Even a slight contamination can permit development in a medium otherwise entirely unsuitable. Hence complete bacterial sterility has been a necessary condition of the experiments. It has at the same time added to the general significance of the results obtained, since in their interpretation there is no need to be concerned with the possible synthetic activities of a microflora either of the intestine or of the environment.

## Methods

*Colony of Aedes aegypti*—This species of mosquito is so adaptable to laboratory conditions that the methods for rearing a stock colony can be

For any one experiment, all the tubes were prepared at the same time and all received the same batch of double strength medium and of cholesterol. Duplicate tubes were usually prepared for each factor being tested, although sometimes three or even five tubes of each were used. Each experiment included control tubes to which no biotin was added, as well as tubes with various known concentrations of biotin. The biotin was added from an autoclaved solution standardized by microbiological assay and stored in the refrigerator. Water-soluble materials to be tested for biotin activity were similarly added from autoclaved stock solutions. Materials insoluble in water, such as oleic acid and the biotin-active oil from hydrolyzed horse plasma (hereafter designated FSF), were emulsified by means of ethyl alcohol. The emulsions were prepared as follows. To 0.95 ml. of warm 95 per cent alcohol in a sterile tube was added 0.05 ml. of the oil, previously autoclaved. 9 ml. of sterile distilled water were then run in with shaking. A milky emulsion formed with some free oil droplets which floated to the surface and were avoided in making the subsequent dilutions. Usually 1 ml. of emulsion was diluted in 9 ml. of sterile water, and the dilute uniform emulsion was added to the experimental tubes in amounts up to 1 ml. The maximal concentration of alcohol was hence only about 0.15 per cent, which was not found to have any effect on growth in the synthetic medium supplemented with biotin. The concentration of active material in the final dilute emulsions was determined by microbiological assay.

In the preparation of an experimental series, the appropriate quantity of sterile distilled water (enough to give a final volume of 6 ml.) was first pipetted into each sterile tube containing casein. This was followed by 3 ml. of the double strength medium and 0.1 ml. of the cholesterol suspension. The biotin or other supplements were then added and the tubes were inoculated with the washed first instar larvae. The usual precautions to insure sterility were observed. The tubes were kept in an incubator at 28–29°, except for a brief period each day when they were removed for examination.

*Microbiological Assays*—Biotin and the biotin activity of lipide materials were assayed with *Lactobacillus casei* with the method of Landy and Dicken (20) slightly modified (2).

*Sterility Tests*—Since most of the media used were clear, nearly colorless liquids, contamination could often be detected by the appearance of cloudiness. All tubes after about 7 days of incubation were submitted to a routine sterility test. This consisted of streaking a loopful of material from each tube onto a plate of dextrose nutrient agar. All tubes which showed some new effect, or the results of which would be especially significant, were later submitted to additional sterility tests in nutrient broth,

yeast extract agar, and cooked meat medium. Contaminations were detected in about 1 per cent of the tubes. As soon as an experimental tube showed evidence of being contaminated it was discarded and the results previously obtained with it were not used.

*Quantitative Expression of Developmental Rate*—The larvae of *Aedes aegypti* undergo three molts between hatching from the egg and pupation. Hence there are four larval instars. These provide the basis for a simple method for following the rate of development. One needs only to examine each tube daily and to note the number of larvae in each instar. Although



FIG. 1. *Aedes aegypti* larvae 7 days after their inoculation as first instar larvae into (from left to right) (a) the basal medium without added biotin (all in the second instar); (b) the basal medium plus 4.2  $m\gamma$  of biotin per ml. (all in the third instar); (c) the basal medium plus 33.3  $m\gamma$  of biotin per ml. (all in the fourth instar). Photograph by J. A. Carlile.

the larvae grow in body length between molts, their head and anal siphon show a discontinuous growth with a marked increase in size immediately following each molt. This fact makes it possible to recognize at a glance whether a larva is in the first, second, third, or fourth instar (see Fig. 1). This method of following growth was used in the early work on the nutrition of mosquito larvae (15). At that time a growth index was described which was obtained from the expression  $N \times (1/T)$ , in which  $N$  is the percentage of larvae reaching the fourth instar within a chosen period of time and  $T$  is the average time in days required by them to do so. The choice of the time period does not materially affect the relative results. A longer period permits a larger percentage to reach the fourth instar in

nearly adequate media, but, since they take longer to do so, the final result is but little changed. For the present work a time of 14 days was chosen, since in this period very few or none of the larvae in the medium without added biotin reached the fourth instar, whereas in the presence of sufficient biotin all reached the fourth instar. Therefore all tubes were examined daily for the first 14 days in order to secure the data for the calculation of  $N \times (1/T)$ . They were then examined every other day for an additional 14 days in order to follow the emergence of adults and to check on the possibility of an unusual delayed growth.

TABLE II

*Effect of Addition of Different Concentrations of Biotin to Synthetic Medium on Growth and Development of Aedes aegypti*

Concentration of added biotin	No. of larvae from group of 6, reaching within 14 days		Adults		Average time to			$N \times \frac{1}{T}$
	3rd instar	4th instar	Female	Male	3rd instar	4th instar	Adult	
<i>mg per ml.</i>					<i>days</i>	<i>days</i>	<i>days</i>	
0	5	0	0	0	10.2			0
6.7	6	6	1	2	5.2	10.3	20	9.7
10.0	6	6		3	4.3	8.0	18	12.5
13.3	6	6	1	1	3.3	8.2	14	12.2
16.7	6	6	2	4	3.5	6.3	14	15.9
33.3	6	5	2	3	3.5	6.4	14	13.0
66.7	6	6	3	3	3.3	4.8	10	20.8
166.7	5	5		3	4.4	7.0	11	11.9

\* In this and the following tables  $N \times (1/T)$  is an expression of the over-all rate of larval development in which  $N$  is the percentage of larvae reaching the fourth instar within 14 days and  $T$  is the average time in days required by these larvae to reach the fourth instar. Under optimal conditions (15) all the larvae reach the fourth instar on the 4th day, so that the maximal value for  $N \times (1/T)$  is 25. Unless otherwise stated, each value is based on the growth of six larvae, three each in two tubes.

### Results

*Biotin and Pimelic Acid*—In the synthetic medium in the absence of added biotin few or none of the larvae attained the fourth instar within 2 weeks and none ever pupated. Biotin concentrations as low as 0.6  $\text{mg}$  per  $\text{ml}$ . had a distinct effect on larval growth, and the value of  $N \times (1/T)$  became progressively higher with increasing concentrations of biotin, until it reached a maximum of 20 in the presence of 66.7  $\text{mg}$  per  $\text{ml}$ . (Tables II and III; Fig. 1). The poorer growth obtained with a still higher concentration of biotin (166.7  $\text{mg}$  per  $\text{ml}$ ., Table II) suggests the possibility of a toxic effect of too much biotin, which has been previously reported for the flour beetle (21). Too few trials have been made at this high level to

permit a definite statement. While a biotin concentration of 50  $\text{m}\gamma$  per ml. or somewhat more was required to give the optimal growth obtainable under the conditions of these experiments, in the presence of only about 20  $\text{m}\gamma$  per ml. most of the larvae metamorphosed into adult mosquitoes, and

TABLE III

*Replacement of Biotin in Larval Growth of Aedes aegypti by Oleic Acid, FSF, and Two Partially Purified Fractions from FSF, Tween 80 and Tween 40*

Supplement	Concentration per ml.		$N \times \frac{1}{T}^*$
	Actual $\text{m}\gamma$	As $\text{m}\gamma$ biotin activity for <i>L. caset</i>	
None	0	0	0.8
Biotin	0.6	0.6	2.5
	2.1	2.1	1.9
	6.2	6.2	6.9
	21.0	21.0	14.3
	2,400	0.02	3.6
Oleic acid	4,800	0.04	4.2
	7,200	0.06	4.6
	3,500	0.007	4.0
FSF	7,000	0.014	6.3
	10,500	0.021	6.4
	3,750	0.015	1.5
Cold alcohol-soluble fraction from FSF	5,000	0.02	3.5
	6,250	0.025	3.3
	1,200	0.01	2.6
Ammoniacal eluate from aluminum oxide adsorption of FSF	1,800	0.015	8.5
	2,400	0.02	4.4
	67,000	0.067	4.3
Tween 80	134,000	0.134	4.7
	333,000	0.333	2.5
	50,000	0.001	0
	100,000	0.002	0
	200,000	0.004	4.3
" 40	400,000	0.008	6.1

\* The figures for no supplement, biotin, oleic acid, and FSF are each based on nine larvae, while those for the other supplements are each based on six larvae.

even with as little as 5 to 6  $\text{m}\gamma$  per ml. a few succeeded in reaching the adult stage. With lower concentrations of biotin the larvae failed to pupate.

Table II shows the adequacy of the single number  $N \times (1/T)$  as an index of larval development. A similar figure could be calculated for the third instar and the same relative values would be obtained in most instances. All larvae reached the second instar on the 2nd or 3rd day regardless of the

presence of added biotin or other supplements unless inhibitory substances were present, in which case they might remain in the first instar for a week or longer before dying. It is also evident from Table II that the value of  $N \times (1/T)$  gives some indication of the extent of metamorphosis to the adult stage. In general, with  $N \times (1/T)$  values of about 15 most of the larvae became adults in about 2 weeks. With  $N \times (1/T)$  values around 20, which is the highest yet observed in the synthetic medium and is close to the maximal possible value of 25, all or most of the larvae became adults in 10 to 12 days. When  $N \times (1/T)$  was 10 or less, only a few or none of the larvae succeeded in reaching the adult stage within the observation period of 4 weeks. A longer period of observation would have been of little value for larvae which reached the fourth instar but failed to pupate within 4 weeks usually began to show signs of weakness at about that time.

Pimelic acid can apparently replace biotin in the nutrition of the flour beetle *Tribolium confusum* (22), serving presumably as a precursor of biotin (23-25). Since *Tribolium* is the only multicellular animal which has been reported as capable of converting pimelic acid to biotin, an attempt was made to discover whether *Aedes aegypti* could also do this. The results of several experiments have been negative. For example, the same experiment in which the results with biotin are given in Table II included the testing of two different samples of pimelic acid, each at concentrations of 3.3, 6.7, 10.0, 13.3, 16.7, 33.3, 66.7, and 166.7  $\text{mg}$  per  $\text{ml}$ .  $N \times (1/T)$  was 0, except in the highest concentration of one sample, for which it was 1.5. There was no significant acceleration of growth over that observed in the tubes without any supplement. It must be concluded that either *Tribolium* has greater synthetic powers than *Aedes*, or its ability to utilize pimelic acid depended on the synthetic activities of microorganisms present in the intestine of the beetle or in the diet, since the experiments with *Tribolium* were not carried on under aseptic conditions.

*Replacement of Biotin by Oleic Acid, FSF, and Related Substances*—Both oleic acid and the oil from hydrolyzed plasma (FSF), when added in place of biotin, had a definite effect on larval growth (Table III). It was important to choose an appropriate range of concentrations. Growth stimulation failed to occur if the concentration was either too low or too high. In the latter case, the materials probably exerted a toxic effect, for with the somewhat higher concentrations most of the larvae remained in the first instar and with still higher concentrations they were killed within 1 day. The range of effectiveness of oleic acid was smaller than that of FSF and the maximal value of  $N \times (1/T)$  obtained in all the experiments in which the two materials were compared was smaller for oleic acid than for FSF. With FSF itself and some active fractions derived from it, values

for  $N \times (1/T)$  of up to 10 to 11 have been frequently obtained, but the highest ever observed for oleic acid was 6.8, and this in only one experiment. Both oleic acid and FSF, if measured in terms of their microbiological biotin activity, would seem to be much more active for mosquito larvae than biotin itself (see, for example, Table III). They are of course much less active if measured in terms of their actual concentration.

Several partially purified fractions which were obtained from FSF (6) and which had biotin activity for *Lactobacillus casei* also had biotin activity for *Aedes aegypti* larvae. The data obtained with two such fractions are given in Table III. The results with the ammoniacal eluate are of special interest, since this material has also been found to have biotin-like activity when injected into chicks on an egg white diet and to have almost as high a specific activity for *Lactobacillus casei* as has oleic acid (6).

A variety of synthetic detergents, chiefly non-ionic esters of fatty acids, has been found to function as growth factors for certain bacteria (26, 5) and to be capable, like oleic acid and FSF, of replacing biotin in the nutrition of lactic acid bacteria (4). Two of these materials, Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) and Tween 40 (a similar monopalmitate), have been tested and found to have some activity for the growth of *Aedes aegypti* when added to a biotin-free medium (Table III).

As might have been expected, lecithin can replace oleic acid for those bacteria for which the latter substance has a growth-promoting effect (5). It can similarly replace biotin in the growth of *Aedes aegypti* (Table IV). At concentrations higher than those shown in Table IV animal lecithin became progressively more and more inhibitory, although even at very high concentrations it did not kill the larvae, which lived for a week or 2 in the first or second instar. Like oleic acid and FSF, lecithin, when used in place of biotin, had a much greater effect on the growth of *A. aegypti* larvae than would be expected from its biotin activity for *Lactobacillus casei*. Thus a concentration (15  $\gamma$  per ml.) with a biotin activity for *L. casei* of only 0.023 m $\gamma$  per ml. gave as high a value for  $N \times (1/T)$  as was usually obtained with biotin concentrations of 4 to 6 m $\gamma$  per ml. This same fact is illustrated by the activity of lecithin when added as a supplement to a low concentration of biotin (Table IV). The addition of 5  $\gamma$  per ml. of lecithin, which theoretically increased the biotin activity for *L. casei* from only 4.2 to 4.208 m $\gamma$  per ml., raised the  $N \times (1/T)$  value from 7 to 15 and permitted the emergence of some adults. The higher concentrations of lecithin, when added to this low concentration of biotin, had less effect, which was the reverse of the situation with lecithin alone. Other experiments have similarly indicated the possibility of an optimal ratio between

biotin and lecithin but more work will have to be done to establish this. It is interesting that lecithin was very early found to affect the growth of *Drosophila* under sterile conditions (27).

In the many experimental tubes with different concentrations of oleic acid and FSF, pupation has never occurred. Among the comparatively fewer tubes containing lecithin at a suitable concentration a viable pupa has been noted in two different experiments. Both pupae died without completing transformation to the adult stage. Since complete tests of the sterility of the tubes did not reveal any contamination, it seems likely

TABLE IV

*Effect of Lecithin in Absence of Biotin and in Presence of Low Concentration of Biotin on Growth of Aedes aegypti*

Supplement	Concentration <i>mg per ml.</i>	$N \times \frac{1}{T}$	Adults	
			Female	Male
None	0	2.6	0	0
Lecithin*	5,000	5.2	0	0
	15,000	9.0	0	0†
	30,000	12.1	0	0
	4.2	7.2	0	0
Biotin† + lecithin	5,000	15.0	3	0
	15,000	13.4	0	1
	30,000	8.3	2	1

\* The lecithin used had a biotin activity for *Lactobacillus casei* of 1.5  $\mu\text{g}$  per  $\text{mg}$ . or 0.0075  $\mu\text{g}$  per 5000  $\mu\text{g}$ .

† Each tube contained biotin at 4.2  $\mu\text{g}$  per  $\text{ml}$ . + the indicated concentration of lecithin.

‡ One larva pupated on the 26th day but failed to emerge as an adult.

that lecithin or some impurity in it is a more suitable source of lipide to replace biotin than either oleic acid or FSF.

#### DISCUSSION

*Aedes aegypti* is now the second insect, and also only the second multicellular animal, to be reared from egg to adult in the absence of microorganisms and on a diet of essentially known composition, *Drosophila melanogaster* having been the first (18). For *Aedes* as for *Drosophila*, the synthetic medium is not quite adequate. More rapid growth and more vigorous adults are regularly observed in sterile media containing certain natural foodstuffs. The unknown factor for both *Drosophila* (18) and *Aedes* (17) is present in the water-insoluble fraction of yeast, and that for *Drosophila* at least is also present in water-soluble form in yeast autolysate (28).



The growth of *Aedes aegypti* in the complete synthetic medium is nevertheless sufficiently good to permit the evaluation of the rôle of individual metabolites in the growth of this insect. It has been possible to show that biotin is essential for the development of *A. aegypti*, as it is for other insects (21, 29), and to determine quantitatively the requirements of this insect for biotin. For *Tribolium confusum* the optimal concentration of biotin was found to be 100 m $\gamma$  per gm. of diet, with as little as 6 m $\gamma$  per gm. producing detectable effects (21). For *A. aegypti* the optimal concentration lies around 50 m $\gamma$  per ml. of liquid medium, with detectable effects at concentrations down to 0.6 m $\gamma$  per ml.

In the presence of casein, sucrose, nucleic acid, and all the known B vitamins, including biotin, *Aedes aegypti* does not require any fat-soluble growth factor other than cholesterol. In this respect it resembles *Drosophila* and several species of beetles (7) and differs from the moths of the genus *Ephesia*, which require linoleic acid even on a diet containing yeast (30). The growth-stimulating effects, in a medium essentially free from biotin, of oleic acid, FSF, lecithin, and related compounds on the larvae of *A. aegypti* may be taken to indicate that biotin functions in the synthesis of such fatty compounds. If suitable lipides are present in the diet, biotin may be largely dispensed with. Such an explanation has been suggested for the ability of some lactic acid bacteria to dispense with biotin in the presence of oleic acid (5) and has been discussed in relation to the biotin-like activity in chicks of the neutral oil from plasma (6). The best larval growth of *A. aegypti* which has been obtained with oleic acid in place of biotin has not been as good as that supported by the lowest concentration of biotin which sufficed for the metamorphosis of some of the larvae to the adult stage. However, with FSF and fractions derived from it, and with lecithin, at appropriate concentrations the rate of larval development has been about as great as the minimum which, in the presence of low concentrations of biotin, was compatible with the occasional emergence of adults. And yet no metamorphosis occurred, with the exception of the two pupae observed in tubes containing lecithin. It may be merely that the lipides, because of their toxic effects, cannot be provided in the nutrient medium at a concentration high enough to carry the larvae through into metamorphosis. Or it may be that biotin is essential also for the synthesis of some particular lipid or other type of compound which has not been provided in the experiments reported here and which is required for metamorphosis but not for larval growth.

#### SUMMARY

The yellow fever mosquito *Aedes aegypti* has been grown from the egg to the adult stage in a medium free from microorganisms and of essentially

known composition. If biotin was omitted from the medium, larval growth was very slow and metamorphosis to the adult stage did not occur. Optimal growth on the synthetic medium was slightly inferior to that observed in liver extract plus heat-killed yeast and was obtained in the presence of about 50 m $\gamma$  of biotin per ml. of medium. With lower concentrations of biotin growth was progressively slower, and with less than about 5 m $\gamma$  per ml. metamorphosis to the adult stage did not take place, although clearly recognizable effects on larval growth were produced by concentrations as low as 0.6 m $\gamma$  per ml. Biotin could not be replaced by pimelic acid.

Relatively low concentrations of oleic acid, an oil from hydrolyzed plasma (FSF), lecithin, and related compounds, when used in place of biotin, supported larval growth as good as that obtained with the lower effective concentrations of biotin.

For the mosquito *Aedes aegypti*, as well as for some bacteria and for chicks, certain lipide compounds can at least partially replace biotin, suggesting that biotin must be of general importance in the synthesis of these lipides.

It is a pleasure to acknowledge the faithful and efficient technical assistance of Miss Marion Orcutt and Mrs. Anne Baldino.

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# TISSUE PROTEINS AND CARCINOGENESIS

## I. THE EFFECT OF CARCINOGENIC AZO DYES ON LIVER PROTEINS

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(Received for publication, August 11, 1948)

Certain constituents of the liver undergo changes which accompany the feeding of the carcinogenic azo dyes. Hoch-Ligeti (1) noted that the activity of the enzyme, succinoxidase, is reduced in livers of rats fed *p*-dimethylaminoazobenzene. Liver tumors induced by this carcinogen have lower cytochrome oxidase and succinoxidase activities than normal liver (2). The azo dyes also lower liver riboflavin in proportion to the carcinogenic activity of the dye (3-5). Recently Miller and Miller (6) demonstrated a firm binding of azo dye to liver proteins before actual tumor formation. Other workers (7, 8) have observed that hepatomas induced by the azo dyes contain more desoxyribonucleic acid than normal tissues, while the ribonucleic acid remains normal or is slightly reduced. Of equal importance are the observations of Price, Miller, and Miller (9) and Masayama and Yokoyama (10) that the precancerous livers of rats fed diets containing *p*-dimethylaminoazobenzene exhibit increased levels of desoxyribonucleic acid. Opie (11) found definite changes in the cytoplasmic ribonucleic acids of liver cells when tumors were produced by the feeding of *p*-dimethylaminoazobenzene.

In the present study livers of rats were fractionated in order to isolate the desoxyribonucleoproteins, ribonucleoproteins, albumins, and globulins. Analyses were made for these components as well as for nitrogen, phosphorus, and riboflavin in the various fractions obtained. The fractionation and analytical studies were carried out on normal rat liver, precancerous liver, and hepatomas resulting from the feeding of diets containing *m'*-methyl-*p*-dimethylaminoazobenzene; this is one of the most active carcinogens of the azo dye group (12).

### Methods

Male albino rats,<sup>1</sup> weighing approximately 200 to 250 gm., were fed diets containing 0.06 per cent *m'*-methyl-*p*-dimethylaminoazobenzene (*m'*Me-DAB) *ad libitum*, for periods up to 10 weeks. The purified basal diet was similar to that used by the Wisconsin group (13), and contained

<sup>1</sup> Holtzman, Sprague-Dawley strain.

casein 18 per cent, glucose monohydrate 73 per cent, corn oil (Mazola) 5 per cent, salts mixture<sup>2</sup> 4 per cent. To each kilo of the diet so prepared were added thiamine hydrochloride 3.0 mg., riboflavin 2.0 mg., calcium pantothenate 7.0 mg., pyridoxine hydrochloride 2.5 mg., and choline 0.5 gm. Each animal was given 2 to 3 drops of halibut liver oil by a dropper at 2 week intervals.

The animals were anesthetized with ether and the livers perfused *in situ* with cold 0.14 M NaCl. The livers from five to six rats usually constituted one group for the fractionation and analytical procedures. The excised livers were rapidly weighed and samples removed for moisture determinations. To the remaining liver were added 3.5 parts by weight of neutral 0.4 M NaCl and the mixture was homogenized for 2 minutes in a Waring blender. After 10 to 15 minutes of stirring, this homogenate was centrifuged for 15 minutes at 4000 R.P.M. (International centrifuge No. 2, conical head). The supernatant fluid was removed and the residue again extracted with a volume of 0.4 M NaCl equal to that of the above supernatant fluid. Following this extraction the remaining residue was stirred with 1.5 volumes of 1.0 M NaCl and centrifuged for 1 hour at 4000 R.P.M. as above. The total homogenate, the first and second extracts with 0.4 M NaCl, the 1.0 M NaCl extract, and the final residue were all analyzed for desoxyribonucleoproteins (DNP) and ribonucleoproteins (RNP) by the hot trichloroacetic acid method of Schneider (14), for nitrogen (15), phosphorus (16), and riboflavin (17, 18).

Albumin and globulin values were determined by analysis of the initial 0.4 M NaCl extract. The extract was reduced to pH 5.0, allowed to stand for 1 hour, and was then centrifuged for 1 hour at 4000 R.P.M. The supernatant fluid containing the globulins and albumin was dialyzed overnight with running distilled water in a rocking dialyzer. The precipitated globulin was centrifuged off and combined with the globulin fraction obtained by precipitation in 2.3 M ammonium sulfate at pH 7.0. The further addition of solid ammonium sulfate to 4.6 M and adjustment of the pH to 6.5 precipitated the albumin fraction. Analysis for the fractions designated as globulin and albumin was made by a modification of the biuret method of Robinson and Hogden (19) by using the linear relationship of optical density to concentration as obtained from crystalline bovine serum albumin. The entire fractionation procedure was carried out in a room maintained at 1°. Special precautions were taken to maintain this temperature during homogenization, centrifugation, etc.

<sup>2</sup> Salts mixture (parts by weight): NaCl 1470,  $\text{Ca}_3(\text{PO}_4)_2$  2086,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  533, KCl 1680,  $\text{CaCO}_3$  2940,  $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$  206,  $\text{KH}_2\text{PO}_4$  4340,  $\text{MgCO}_3$  672,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  3.2,  $\text{K}_2\text{Al}_2(\text{SO}_4)_3 \cdot 12\text{H}_2\text{O}$  1.2,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  5.4, NaF 7.4, and KI 1.2.

## Results

*Fractionation of Normal Rat Liver*—The initial fractionations were carried out by successive extractions of homogenized rat liver with 0.14 M NaCl as described by Mirsky and Pollister (20). It was difficult, however, to remove more than 40 per cent of the total RNP with this concentration of NaCl. Various combinations of extractions were carried out to improve the method. Three successive extractions with 0.4 M, 0.28 M, and 0.14 M NaCl removed 85 per cent of the total RNP and 8 per cent of the total DNP. It was noted, however, that most of the RNP appeared in the initial 0.4 M NaCl extract. When the extractions were carried out with NaCl

TABLE I  
*Liver Fractionation*

Mg. per 100 gm. of fresh liver.\*

Liver fraction	Nitrogen		Phosphorus		DNP†		RNP‡		Riboflavin	
	mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent of total	mg. per cent	per cent of total	γ per cent	per cent of total
Total homogenate.....	2520		280		266		150		2466	
1st extraction, 0.4 M NaCl.....	1400	56	164	58	19	7	98	65	1007	41
2nd " 0.4 M NaCl.....	258	10	27	10	8	3	18	12	182	7
Extraction, 1.0 M NaCl.....	250	10	39	14	176	66	15	10	206	8
Final residue.....	602	24	52	18	61	23	20	13	1134	46

\* The values expressed are the averages of six fractionations, five or more rats per group. Rats maintained on the basal purified diet 4 weeks. Liver moisture, 74 to 76 per cent. Average daily food intake, 18 gm. per rat; average liver weight, 10.0 gm.

† Desoxyribonucleoprotein as desoxyribonucleic acid.

‡ Ribonucleoprotein as ribose.

solutions of a higher concentration than 0.4 M (*i.e.*, 0.5 to 0.6), slightly more RNP was removed but increasing amounts of the DNP were extracted. The viscosity of the homogenate increased at salt concentrations above 0.4 M, and at 0.6 M the mixture was extremely viscous, indicating solvation of the DNP. From histological data it was apparent that the nuclei, which contain most of the DNP, remained relatively intact up to NaCl concentrations of 0.4 M. These observations, along with analytical data, prompted the use of 0.4 M NaCl for the initial extractions. The complete method has already been described. The values obtained for the different components in the various fractions are presented in Table I.

The initial extraction with 0.4 M NaCl removed over 50 per cent of the total nitrogen and phosphorus, 41 per cent of the total riboflavin, 7 per cent

of the DNP, and 65 per cent of the RNP. All of the soluble globulin and albumin appeared in this extract. Subsequent extraction with 1.0 M NaCl resulted in a fraction containing 66 per cent or more of the liver DNP, 14 per cent of the total phosphorus, and approximately 10 per cent of the total RNP, riboflavin, and nitrogen. The final residue contained 23 per cent of the DNP. This can be removed, if desired, by exhaustive extraction with 1 M NaCl. Riboflavin also appeared to concentrate in this fraction, presumably in combination with the sodium chloride-insoluble protein fraction.

TABLE II

*Effect of Carcinogenic Azo Dye on Liver Proteins*

Purified basal diet + 0.06 per cent *m'*-methyl-*p*-dimethylaminoazobenzene.

Component	Control basal diet	Time rats were fed diet containing azo dye				Hepatic mass
		2 wks.	4 wks.	6 wks.	8 wks.	
Nitrogen, mg. % . . . . .	2520	2470	2470	2500	2465	2040
Phosphorus, mg. % . . . . .	280	316	316	308	326	272
Desoxyribonucleoprotein, as desoxyribonucleic acid, mg. % . . . . .	266	396	440	490	506	756
Ribonucleoprotein, as ribose, mg. % . . . . .	150	122	146	140	126	139
Riboflavin, $\gamma$ % . . . . .	2466	1967	1740	1395	1278	
Albumin, % . . . . .	1.3	1.1	1.2	1.1	1.1	
Globulin, % . . . . .	1.3	2.1	2.0	2.2	2.2	
Non-protein nitrogen, mg. % . . . . .	180	181	213	205	230	
“ phosphorus, mg. % . . . . .	81	98	104	108	108	

The values are expressed on a fresh liver basis and are averages of three or more groups per period, four to six rats per group. Cirrhosis was evident after 4 weeks and at 8 weeks most of the livers were extremely cirrhotic. Liver moisture, 75 to 78 per cent. Food intake while on diet containing azo dye, 13.5 to 15 gm. per rat per day. Average liver weight, 9.7 gm. per rat (range 7.2 to 13.5).

It is apparent that this procedure provides only crude fractions. The methods employed to concentrate and purify certain of the components will be described later.

*Effect of Azo Dyes on Liver Components*—The feeding of diets containing *m'*-Me-DAB appeared to induce some pronounced changes in certain of the liver components (Table II). The total liver moisture remained relatively constant (75 to 78 per cent) over the 8 week feeding period, as did the total nitrogen. The non-protein nitrogen, however, increased somewhat as a result of feeding the azo dye. An increase was observed in the total phosphorus of the liver which could be accounted for by the increase in the nucleic acid fractions. Of all the components determined, the DNP exhibited the greatest change as a result of administration of the



carcinogen. There was a progressive increase, and at 8 weeks this component was almost double that observed in the normal rat liver (266 *versus* 506 mg. per cent). Cirrhosis and other liver damage progressed as the azo dye was fed, and there appeared to be some correlation between increasing liver DNP and cirrhosis. The high liver DNP, after feeding of the carcinogenic diet for 8 weeks, actually approached that observed in hepatomas. It should be noted that the estimation of DNP and also of RNP depends on the colorimetric action of desoxyribose and ribose bound to purine bases after the nucleic acids have been liberated from the protein

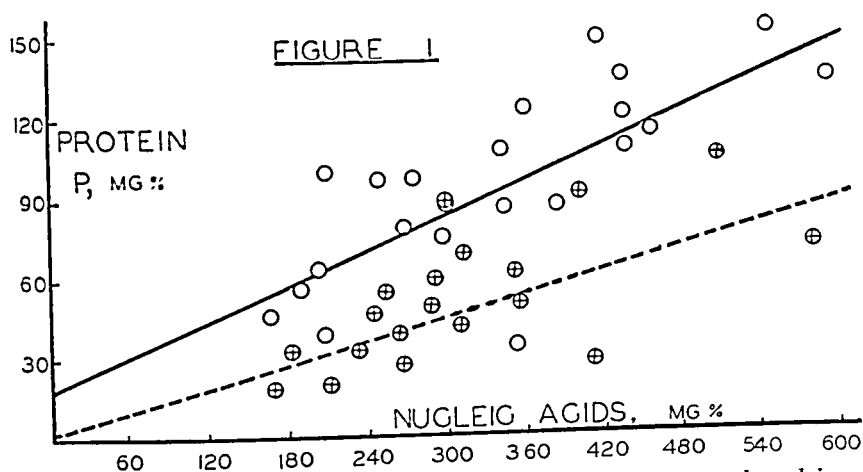


FIG. 1. The protein phosphorus content plotted against the total nucleic acid content of various NaCl extracts of liver, indicating that increases in the amounts of nucleic acids are accompanied by an increased phosphorus content. O, corresponds to the 0.4 M NaCl extract (mostly ribonucleic acid);  $\otimes$ , corresponds to 1.0 M NaCl extracts (mostly desoxyribonucleic acid). The lines are calculated by the method of least squares.

by hot trichloroacetic acid (method of Schneider (14)). An increase in other components that react with the Dische reagent, for example, would result in apparent high values for DNP. It appears improbable that this was responsible for the high values observed in the livers of rats fed the diet containing azo dye, since an actual increase in the quantity of the DNP fraction could be observed visually when this nucleoprotein complex was precipitated by reducing the NaCl concentration in the 1.0 M extract to approximately 0.14 M. Phosphorus data also support this conclusion. The total of nucleic acids as determined colorimetrically in the initial fraction obtained by extraction with 0.4 M NaCl has been plotted against the protein phosphorus content of this fraction for each of the twenty-two runs (Fig. 1). It may be observed that an increase in nucleic acids is cor-

related with an increase in protein phosphorus. Similarly, the phosphorus content of the 1.0 M extract, which contains most of the DNP, was plotted against nucleic acid content. Here again it is evident, from the line of least squares, that increases in nucleic acids are accompanied by an increased phosphorus content. This provided further evidence that the increase in the liver DNP accompanying the feeding of the azo dye is real and not a result of other substances that may react with the Dische reagent.

At present it is difficult to assign any significance to changes in the RNP content (Table II). There was an initial decrease in this component accompanying the feeding of diets containing the azo dye. After feeding the dye for 4 to 6 weeks, however, there was some increase. These observations appear to be in agreement with those of Opie (11), although his studies were on a cytological basis and involved the feeding of the less active carcinogen, *p*-dimethylaminoazobenzene.

The riboflavin content decreased progressively in the liver; after 8 weeks of feeding the diet containing the *m*'Me-DAB, it was approximately 50 per cent of that observed in the livers of rats fed the dye-free basal diet (2466 versus 1278  $\gamma$  per cent). These findings are in agreement with those of other investigators (5, 9). Normal rat liver contains 1.3 per cent globulin and almost the same percentage of albumin. The albumin remained at approximately this level throughout the period of azo dye feeding. An increase was noted, however, in the globulin fraction. This increase appeared within 2 weeks and the globulin content remained at approximately 2 per cent throughout the precancerous period.

In a corresponding study on the effect of azo dye carcinogenesis on serum proteins, it was observed that the serum  $\gamma$ -globulin concentration increased with the feeding of the *m*'Me-DAB.<sup>3</sup> Globulin and albumin were not determined in the liver tumors because of the small amounts of suitable tissue available.

In order to ascertain whether the increase in DNP was specific for the carcinogenic azo dyes or whether this was perhaps a response to azo compounds in general, rats were fed diets that contained the relatively non-carcinogenic compound, azobenzene, at a level of 0.05 per cent (Table III). In this series, the total liver nitrogen and phosphorus contents were somewhat higher than were observed for the basal control diets (Table I). Riboflavin, DNP, and RNP concentrations were essentially normal after the diet containing the azobenzene was fed for either 2 or 4 weeks, indicating that the increase in the DNP content noted in the animals fed the *m*'Me-DAB was associated with carcinogenesis and was not due to azo compounds in general.

<sup>3</sup> Cook, H., Griffin, A. C., and Luck, J. M., *J. Biol. Chem.*, in press.

*Purification of Liver Nucleoproteins*—Extraction of liver with 0.4 M NaCl resulted in a fraction containing 65 per cent of the total liver RNP and approximately 7 per cent of the DNP. The 1.0 M extract contained 66 per cent of the total DNP and 10 per cent of the RNP (Table I). Almost all of the RNP present in the initial NaCl extract may be sedimented out by adjusting the pH to 5.0 and centrifuging. If this precipitate was stirred in water and the pH adjusted to 8.0, the RNP was dissolved. By repeating this process several times, it was possible to obtain a fraction containing over 50 per cent of the RNP originally present in the liver and from 2 to 5 per cent of the total DNP. This fraction was also free of the more soluble liver components; *i.e.*, albumins, globulins, non-protein nitrogen, and phosphorus. By centrifuging the initial NaCl extract for 4 hours

TABLE III  
*Effect of Azobenzene on Liver Components*

Purified basal diet + 0.05 per cent azobenzene.

Component	2 wks.	4 wks.
Nitrogen, mg. %.....	2900	2850
Phosphorus, mg. %.....	342	332
Desoxyribonucleoprotein, as desoxyribonucleic acid, mg. %....	318	273
Ribonucleoprotein, as ribose, mg. %.....	153	170
Riboflavin, $\gamma$ %.....	2330	2278
Non-protein nitrogen, mg. %.....	200	199
“ phosphorus, mg. %.....	100	102

The values are on a fresh tissue basis, two groups per period, four to six rats per group. Livers appeared normal at the 2 and 4 week periods.

at 100,000  $\times g$  in the ultracentrifuge at pH 7.0, an almost complete sedimentation of the RNP was effected. This high speed centrifugation probably sedimented the formed elements of the cytoplasm that contain the RNP (21). Further work is in progress to determine whether ultracentrifugation will aid in the purification of RNP fractions.

Some preliminary steps were also taken toward further purification of the DNP from the 1.0 M NaCl extract. Mirsky and Pollister (20) precipitated the DNP from this fraction by adjusting the NaCl to 0.14 M, at which concentration the protein appeared in a fibrous state. The fibers were then redissolved in 1.0 M NaCl. This procedure, repeated one or more times, resulted in a preparation of DNP relatively free of the RNP and other components. In the present investigation, purification by precipitation in 0.14 M NaCl resulted in a preparation containing approximately 50 per cent of the DNP and 6 per cent of the RNP originally present in the liver. This fraction also contained 4 to 8 per cent of the total liver nitrogen,

approximately 10 per cent of the phosphorus, and had a N:P ratio of 4.0. All the preparations contained some riboflavin, although this constituted only 2 to 3 per cent of the total liver riboflavin in most cases. No studies were made to determine whether a flavoprotein constituted an integral part of the DNP present or whether the riboflavin present was merely residual. A DNP preparation containing relatively little RNP was prepared by precipitation of the fibers in 0.4 M NaCl instead of in physiological saline. Some DNP was lost by precipitation in 0.4 M NaCl because of the increased solubility at this higher salt concentration. The resulting DNP, however, contained less than 2 per cent of the total RNP and had a N:P ratio of 3.8. In this range it becomes difficult to obtain an accurate analysis for the RNP in the presence of large quantities of DNP.

It became evident that a better initial separation of the two nucleoprotein types would facilitate the subsequent purification of these different fractions. It had been observed previously that low temperature storage of liver altered the extractability of certain of the proteins. A quantity of perfused rat liver was divided into three equal portions. One portion was fractionated immediately while the second and third portions were stored at  $-15^{\circ}$  for 2 and 4 weeks respectively. When the tissues stored at the low temperature were extracted with 0.4 M NaCl, more of each component appeared in this fraction. Thus, over 80 per cent of the total nitrogen and phosphorus were extracted, as compared with approximately 55 to 60 per cent of these components in fresh, non-frozen liver (see Table I). Approximately 90 per cent of the RNP was removed after the tissues had been subjected to the low temperature storage as compared to a 65 per cent extraction in non-frozen liver. Perhaps more striking was the removal of riboflavin after the cold treatment. Only 40 per cent or less of the total riboflavin was initially extracted in the non-frozen liver, whereas 90 per cent could be extracted after the liver was frozen for the 2 or 4 week period. Variations as to time of storage, freezing temperature, and methods of freezing were carried out in order to determine whether low temperature would actually improve extractability and the fractionation of the liver proteins. It was found that freezing of the livers for only a few hours at  $-15^{\circ}$  improved the extractability of most components. The same was noted if the liver was frozen in a dry ice-ether (or acetone) mixture for 10 to 15 minutes. Finally a fractionation was carried out wherein the freshly removed liver tissue was immersed in liquid air for 30 minutes. The tissue was then homogenized and twice extracted with 0.4 M NaCl as described under "Methods." The remaining residue was extracted with 6 volumes of 1.0 M NaCl. All centrifugings were made in a Servall supercentrifuge for approximately 20 minutes at  $20,000 \times g$ . The initial extract with 0.4 M NaCl contained 83 per cent of the total RNP and 4 per cent of the DNP,

while the 1.0 M NaCl extract contained 82 per cent of the DNP and 3 per cent or less of the RNP. From these fractions it should be possible to obtain relatively pure preparations of RNP, DNP, albumins, globulins, etc., by the methods previously described.

#### DISCUSSION

The increase in the liver DNP content appeared to be one of the most significant changes accompanying the administration of the carcinogen, *m*'Me-DAB. Other investigators (9) have also observed that a similar response, though less intense, could be obtained when the azo dye, *p*-dimethylaminoazobenzene, was fed. A relatively non-carcinogenic compound, azobenzene, had seemingly little effect on the liver DNP, suggesting that the above increase was only associated with the process of azo dye carcinogenesis. Hepatic tumors have a considerably higher DNP concentration than normal liver tissue, and in the present investigation it was found that the feeding of the carcinogenic azo dye resulted in a progressive increase in the amount of this component to a level approaching that of actual tumor tissue. Generally, it has been observed that tumor tissues contain more DNP than the normal tissues of tumor origin; however, the concentration of nucleic acids varies considerably in different normal tissues (7). Stowell (22) also has observed a higher cell content of desoxyribonucleic acid in epidermoid carcinoma than in normal human epidermis.

Histological studies were not made on the liver of rats fed the azo dyes. It would appear certain that cellular changes accompany the large increase in DNP in the liver cell nuclei. Schneider (23) observed a cytoplasmic nuclear ratio of 5.85 for normal rat liver and 3.04 for rat hepatomas, indicating that the tumor cells have large nuclei or a greater number of small cells with nuclei of approximately the same size as normal cells. Our own observations reveal only an over-all increase in the total liver DNP and provide no information as to whether it is a result of enlarged cell nuclei, of an increased number of small cells, or of an increase in DNP content per unit volume with no change in nuclear or cell size. There was a decrease in the amount of liver riboflavin with the feeding of azo dyes which is also in agreement with the findings of other investigators (3-5). This decrease in liver riboflavin appears to be roughly proportional to the increase that occurs in the DNP. Both components in the precancerous livers approach the levels found in hepatomas, which may be of significance in the actual process of carcinogenesis. It is difficult to compare normal or even precancerous liver with liver tumors without a consideration of the type and uniformity of tissues involved. In the present study, more emphasis was placed on the precancerous liver changes, although it appears significant that certain components of the precancerous livers do approach the levels found in liver tumors.

## SUMMARY

1. The livers from normal rats and from those fed diets containing the carcinogenic azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene, were fractionated into several protein components on a basis of differential solubility in NaCl solutions. Quantitative analyses were made for nitrogen, phosphorus, desoxyribonucleoproteins, ribonucleoproteins, riboflavin, albumin, globulin, and non-protein nitrogen and phosphorus in the appropriate fractions.

2. Methods of purifying the ribo- and desoxyribonucleoproteins are described. Freezing of liver tissue before fractionation improved the extractability of most of the liver proteins.

3. The amount of desoxyribonucleoproteins increased progressively and approached the hepatoma level as the diet containing this azo dye was fed. The normal level was 266 mg. per cent (as desoxyribonucleic acid); after receiving azo dye for 8 weeks it was 506 mg. per cent; the level in liver tumors was 756 mg. per cent. The liver riboflavin content decreased as the azo dye was fed; the globulin content increased significantly, while concentrations of other components remained relatively normal.

4. A relatively non-carcinogenic azo compound, azobenzene, had no appreciable effect on any of the components determined.

We wish to acknowledge with thanks the receipt of grants-in-aid from the American Cancer Society and the United States Public Health Service. The assistance of Eleanore Frey and Carol Moore in conducting many of the analyses incident to these studies is also gratefully acknowledged. The histological studies referred to were very kindly made by Dr. H. Kirkman of this University.

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was soluble only in water, non-aqueous reagents were not employed. The pH values of all inactivation reactions were below 7, most of them being between pH 4.5 and 6.0.

Reagents found to be ineffective as inactivating agents are listed in Table IV.

TABLE II  
*Inactivation Reagents Studied Qualitatively*

Reagent	Conditions for inactivation of >50%
Potassium dichromate.....	0.005 M, 37°, 3 hrs.
Ferric chloride.....	0.001 " 97°, 10 min.
Hydrogen peroxide.....	30% solution, diluted 1:1000, 97°, 6 min.
Hydroxylamine hydrochloride...	0.001 M, 97°, 15 min.
Sodium nitrite*.....	0.005 " 25°, 3 hrs.
Nitranilic acid.....	0.01 " 97°, 10 min.
Auric chloride†.....	4%, 25°
Chloroplatinic acid.....	10%, 25°
Mercuric sulfate‡.....	10% in H <sub>2</sub> SO <sub>4</sub> , 25°
Norit§.....	25°, 5 min.
Amberlite IR-100-H§.....	25°, 5 "

\* Reagent caused vasodilatation.

† Inactivation performed on pure serotonin.

‡ The activity was precipitated. Recovery after removal of Hg<sup>++</sup> with H<sub>2</sub>S has not exceeded 20 per cent.

§ These adsorbents are listed because both are effective in removing activity, none of which has ever been eluted under a wide variety of conditions.

TABLE III  
*Inactivating Effect of pH on Purified Serum Vasoconstrictor at 97°\**

pH	Time	Per cent inactivation
	<i>min.</i>	
4.5-6.5	360	0
1.5	360	0
0.5	240	30-40
7.5	30	20
10.3†	10	>90

\* Under nitrogen.

† No inactivation in preceding 30 minutes at 25°.

## DISCUSSION

Inactivation studies are difficult to appraise as evidence for the existence of groupings. It is clear, as has been pointed out (2), that a posi-

ear preparation was then compared as described (1) with controls containing no reagent. No loss of activity was observed in the controls. Reagent controls were performed; it was found that, at the concentrations employed, most of them did not exert significant effects.

With several reagents examined according to this scheme, the extent of inactivation was directly proportional to the quantity of reagent employed, while with others it was not (Table I). With still others, only qualitative observations were made, either because the reagents could not be adapted

TABLE I  
*Inactivation Reagents Studied Quantitatively*

97°, 5 minutes, pH 4 to 6.

Reagent	Concentration	Quantity to in- activate 100 units of purified vasoconstrictor
	<i>moles per l.</i>	<i>micromole</i>
Potassium persulfate.....	$4 \times 10^{-5}$	0.006
Iodine*.....	$5 \times 10^{-5}$	0.006
Potassium permanganate†.....	$5 \times 10^{-5}$	0.005
Sodium bisulfite.....	$1 \times 10^{-4}$	0.02
Pyridine perbromide hydrobromide.....	$5 \times 10^{-4}$	0.04
Potassium ferricyanide.....	$5 \times 10^{-4}$	0.06
Chloramine-T.....	$5 \times 10^{-4}$	0.15
Ceric sulfate†.....	$1 \times 10^{-4}$	~0.01
Potassium periodate†.....	$1 \times 10^{-4}$	~0.02
Iodic acid†.....	$5 \times 10^{-4}$	~0.05

\* 3 minutes; 25°, 60 minutes.

† 25°, 10 minutes.

‡ Extent of inactivation not proportional to quantity of reagent.

to the method described or because they did not appear to be sufficiently effective (Table II).

The three most effective inactivating reagents have been tried on pure serotonin. Under closely similar conditions, within an experimental error of 20 per cent, the same quantity of iodine (or potassium persulfate) was required to inactivate 100 units of either the concentrate or pure serotonin. With potassium permanganate, twice as much reagent was required with the less pure material.

*Effect of pH*—The inactivating effect of alkali was found to be much more rapid than that of acid (Table III). The results obtained with acid were variable until the experiments were performed under nitrogen.

Because of the sensitivity of the serum vasoconstrictor to alkali, strongly alkaline reagents were not tried. Moreover, since the purified preparation

was soluble only in water, non-aqueous reagents were not employed. The pH values of all inactivation reactions were below 7, most of them being between pH 4.5 and 6.0.

Reagents found to be ineffective as inactivating agents are listed in Table IV.

TABLE II  
*Inactivation Reagents Studied Qualitatively*

Reagent	Conditions for inactivation of >50%
Potassium dichromate.....	0.005 M, 37°, 3 hrs.
Ferric chloride.....	0.001 " 97°, 10 min.
Hydrogen peroxide.....	30% solution, diluted 1:1000, 97°, 6 min.
Hydroxylamine hydrochloride...	0.001 M, 97°, 15 min.
Sodium nitrite*	0.005 " 25°, 3 hrs.
Nitranilic acid.....	0.01 " 97°, 10 min.
Auric chloride†.....	4%, 25°
Chloroplatinic acid.....	10%, 25°
Mercuric sulfate‡.....	10% in H <sub>2</sub> SO <sub>4</sub> , 25°
Norit§.....	25°, 5 min.
Amberlite IR-100-H§.....	25°, 5 "

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## DISCUSSION

Inactivation studies are difficult to appraise as evidence for the existence of chemical groupings. It is clear, as has been pointed out (2), that a posi-

tive result indicates change in the structure of the molecule, while lack of inactivation does not necessarily preclude such change. When impure preparations are employed, an additional difficulty arises; namely, inactivation may be due to a product of the reaction between the reagent and an impurity. Even more ambiguous is the reasoning that certain reagents react *only* with specific chemical groupings. Carrying out the inactivation studies on a quantitative basis, and in such a way that minimal handling was required before bioassay, made it easier to evaluate the findings.

It is believed, on the basis of the results so far obtained, that serotonin has a sufficiently sensitive structure to permit most "classification" re-

TABLE IV  
*Reagents Causing No Inactivation under Conditions Employed*

Reagent	Conditions
Ferrous sulfate	0.005 M, 97°, 10 min.
Silver nitrate	0.005 " 97°, 10 "
Ninhydrin*	0.005 " 97°, 12 "
Formaldehyde	40% solution, diluted 1:1000, 97°, 12 min
Cupric sulfate	0.001 M, 97°, 10 min.
Cuprous oxide†	25°
p-Chloromercuribenzoic acid‡	25°
Diazomethane§	25°
2,4-Dinitrophenylhydrazine	Suspension, 37°, 90 min.
Amberlite IR-4B	25°, 60 min.

\* Under these conditions, 30 per cent inactivation of a sample of pure serotonin was observed. The reagent has a weak constrictor effect.

† Precipitate formed. The supernatant retained full activity.

‡ Kindly supplied by Dr. T. P. Singer, Western Reserve University.

§ Performed in methanolic solution on pure serotonin. Nitrogen liberated; no activity lost.

agents to cause inactivation under relatively mild conditions. However, because of this sensitivity to such a variety of reagents at relatively high dilutions, it does not seem justifiable as a result of these studies alone to draw conclusions as to the presence of specific chemical groupings.

Conditions may ultimately be found under which the reagents in Table I will cause relatively specific inactivation of serotonin. This requires determination of the mildest conditions which are still effective. For example, it has been found that iodine is as effective at room temperature as at 97°, although the minimal time requirement, which is less than 1 hour, must still be determined.

The fact that iodine completely destroys the activity, and that the same quantity of iodine destroys the same number of units of activity of both

pure serotonin and the concentrate only 0.75 per cent pure,<sup>2</sup> indicates that the total activity of the impure preparation is due, probably, to a single substance, namely serotonin. Additional evidence comes from the correlation of activity and color produced in the Ehrlich reaction<sup>3</sup> in the course of further purification. If the sensitivity of the serotonin molecule is considered in conjunction with its relatively high (25 to 35 per cent) recovery from serum in the concentrate, the conclusion seems justified that the vasoconstrictor property of serum, at least as measured in the rabbit ear preparation, is almost entirely due to serotonin.

The authors wish to acknowledge the valuable assistance of Mr. John M. Means and Miss Elizabeth Hunt in this investigation.

#### SUMMARY

In order to determine which reagents were most suitable for abolishing the vasoconstrictor activity of serum, the inactivation of a beef serum vasoconstrictor (serotonin) concentrate by chemical reagents was studied. Certain oxidizing and halogenating agents, especially potassium persulfate, potassium permanganate, and iodine, were found to be effective at high dilution. By comparing the quantity of iodine required for inactivation of the concentrate and pure serotonin, evidence was obtained favoring the view that serotonin is solely responsible for the vasoconstrictor activity of serum.

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<sup>2</sup> The activity of pure serotonin is 100,000 units per mg. The concentrate contains 750 units per mg.

<sup>3</sup> Unpublished data (see Paper IV for the method).

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Cupric sulfate	0.001 M, 97°, 10 min.
Cuprous oxide†	25°
p-Chloromercuribenzoic acid‡	25°
Diazomethane§	25°
2,4-Dinitrophenylhydrazine	Suspension, 37°, 90 min.
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pure serotonin and the concentrate only 0.75 per cent pure,<sup>2</sup> indicates that the total activity of the impure preparation is due, probably, to a single substance, namely serotonin. Additional evidence comes from the correlation of activity and color produced in the Ehrlich reaction<sup>3</sup> in the course of further purification. If the sensitivity of the serotonin molecule is considered in conjunction with its relatively high (25 to 35 per cent) recovery from serum in the concentrate, the conclusion seems justified that the vasoconstrictor property of serum, at least as measured in the rabbit ear preparation, is almost entirely due to serotonin.

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<sup>2</sup> The activity of pure serotonin is 100,000 units per mg. The concentrate contains 750 units per mg.

<sup>3</sup> Unpublished data (see Paper IV for the method).



# SERUM VASOCONSTRICTOR (SEROTONIN)

## IV. ISOLATION AND CHARACTERIZATION\*

By MAURICE M. RAPPORT,† ARDA ALDEN GREEN, AND IRVINE H. PAGE

*(From the Research Division of the Cleveland Clinic Foundation, Cleveland)*

*(Received for publication, July 17, 1948)*

In the first paper of this series (1) the partial purification of a vasoconstrictor substance from beef serum was reported. Further purification of the active substance led to its isolation. A preliminary report of the method together with analytical and pharmacological data has also been published (2). It is the purpose of this paper to present the details of the method of isolation, the more complete analyses now at hand, and some further characterizations of the substance.

The problem of isolating this principle presented the difficulties usually encountered in vitamin research (3) in that the substance was present in the source material (beef serum) in a dry weight concentration of about 0.005 per cent. Initial obstacles were overcome with the preparation of a stable concentrate which was 0.8 per cent pure and contained 25 to 35 per cent of the activity originally present in the serum (the percentages are calculated from the activity of the pure substance).

This concentrate was composed mainly of the ammonium salt of 5-nitrobarbituric acid (the precipitating agent). The active principle appeared to be involved in this concentrate in some kind of complex, since repeated recrystallization did not effect important changes in activity of the crystals so obtained. After many procedures which resulted in large losses of activity were tried, it was found possible to separate 90 per cent of the inactive materials from the concentrate with a loss of less than 5 per cent of the total activity by precipitating them with acetone from hot aqueous solution. Concentration of the filtrate and recrystallization of the residue from methyl alcohol led to the isolation of a crystalline substance with vasoconstrictor action in the rabbit ear preparation approximately twice that of commercial epinephrine.

### EXPERIMENTAL

Melting points were determined on the Kofler micro hot stage and are corrected.

\* This study was supported in part by a grant from the United States Public Health Service, Cardiovascular Study Section.

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*Isolation*—28 gm. of concentrate, prepared as described (1) from about 210 liters of beef serum (450 liters of blood) and containing 750 units<sup>1</sup> of activity per mg., were dissolved in 3.4 liters of boiling distilled water. 3.5 liters of reagent grade acetone were then added to the hot (80°) solution, resulting in the immediate formation of a copious, finely divided precipitate.

For convenience of operation, especially with regard to minimizing the time of heating and reducing the hazards attending the addition of the acetone to a solution whose temperature was above the boiling point of acetone, this procedure was carried out in separate portions by employing one-seventh the quantities listed above, which were then combined.

After chilling the mixture in the cold room overnight, the colorless precipitate was filtered by suction and washed with 50 per cent aqueous acetone. This precipitate weighed 24.0 gm. and assayed at 25 units per mg. The filtrate was then evaporated below 50° at reduced pressure under nitrogen to a volume of 245 ml. The precipitated material resulting from this evaporation was dissolved by boiling the solution, the addition of 55 ml. of water being required. 350 ml. of acetone were then added, the mixture was left in the cold room 2 days, and then suction-filtered, giving 1.64 gm. of buff-colored precipitate assaying at 200 units per mg. The filtrate (containing 18 million units) was then evaporated to dryness as before. The residue was extracted with 50 ml. of 50 per cent aqueous methanol. This extract was evaporated to dryness, and the residue was extracted with 10 ml. of hot absolute methanol. On cooling, this extract deposited brownish yellow rosettes of prisms. The supernatant was decanted, and the crystals were washed with methanol and acetone by decantation. After drying the crystals weighed 143 mg., m.p. 196–201° (decomposition); assay 80,000 units per mg.

The crystals were recrystallized by dissolving them in 10 ml. of water and, at 60°, adding 35 ml. of acetone, giving, after washing and drying, 108 mg. of light buff-colored, thin rhomboid plates, m.p. 207–212° (decomposition); assay 100,000 units per mg. By adding 50 ml. of acetone, a second crop of 12.5 mg., m.p. 201–205° (decomposition), was obtained from the filtrate.

The first crop material was again recrystallized from 6 ml. of water and 18 ml. of acetone to give 93 mg. of light buff platelets, m.p. 209–212° (decomposition); assay 100,000 units per mg. By adding 75 ml. of acetone to the mother liquor, a second crop of 9.5 mg., m.p. 204–208° (decomposition), was obtained.

<sup>1</sup> The unit of activity has been defined (1) as the response given in the rabbit ear preparation by 0.2 ml. of a solution containing 1.2  $\gamma$  per ml. of a purified serum vasoconstrictor preparation.

The result described above represents the last "large scale" isolation effort and is the best one. The procedure has been carried out twice on the scale described, and several other times with about one-tenth this quantity of concentrate. In each experiment, the pure active substance has shown the same physical and chemical properties. One of the two complications encountered in the less straightforward experiments was the presence of an impurity which had the same solubility characteristics in water, methanol, and acetone as the active substance and appeared to be inorganic, with a melting point near 290°. The other was the formation of diliturate complexes of variable composition (as revealed by ultraviolet absorption spectra) which were deposited by the methanol extract and could not be substantially purified by several recrystallizations from methanol.

Variations from the procedure described which were employed in the previous experiments may have contributed to their less satisfactory results. Lack of additional concentrate prevented clarification of this point.

*Melting Point*—This is a decomposition point, slight effervescence occurring at and slightly above it. The discoloration of the sample is slight and the melt is clear. The sharpest decomposition point, obtained on the first analytical sample, was 212–214°. The same sample, after 3 weeks, melted at 206–209°. The melting points were taken by a uniform procedure. Heating was begun below 50°, carried to within about 20° of the melting point at 6–8° per minute, and at the decomposition point the rate of heating was 2–2.5° per minute.

*Elementary Analyses*—Two samples, prepared from different batches of concentrate, were analyzed by two analytical laboratories.

$C_{14}H_{21}O_3N_5S$ (405.4).	Calculated.	C 41.47, H 5.72, N 17.28, S 7.91, N-CH <sub>3</sub> 3.71
Sample I, m.p. 212–214° <sup>2</sup>	Found.	" 41.38, " 6.03, " 16.97
" II, " 209–212° <sup>3</sup>	"	" 41.75, " 5.87, " 17.63, S 8.03, N-CH <sub>3</sub> 2.99

An analysis for ionic sulfate, performed according to the nephelometric procedure of Hoffman (4) on 0.50 and 0.75 mg. of Sample II, gave 21 and 23 per cent sulfate.  $C_{14}H_{21}O_3N_5 \cdot H_2SO_4$  requires 23.7 per cent. The isolated material thus appears to be the sulfuric acid salt of an organic base.<sup>4</sup>

*Solubility*—Serotonin is soluble in water to the extent of 100 mg. per ml. at 50° and about 20 mg. per ml. at 27°. The substance is also soluble in glacial acetic acid. The pure material is very sparingly soluble in metha-

<sup>2</sup> Analyses by E. Thommen, Basel.

<sup>3</sup> Analyses by A. Elek, Los Angeles.

<sup>4</sup> The name "serotonin" which was proposed for the isolated substance (2) should properly be reserved for the free base. The isolated material would then be serotonin sulfate. Since only the sulfate is considered in this communication, serotonin, when used, refers to the sulfate.

nol and 95 per cent ethanol, and insoluble in absolute ethanol, acetone, pyridine, chloroform, ethyl acetate, ether, and benzene.

*Optical Activity*—A solution of 10.4 mg. of serotonin in 1.98 ml. of water showed no significant rotation at 31° in a 2 dm. tube.

*Ultraviolet Absorption Spectrum*—The absorption spectrum of serotonin in aqueous solution at pH 3.5 has a maximum at 2750 Å, a shoulder with a

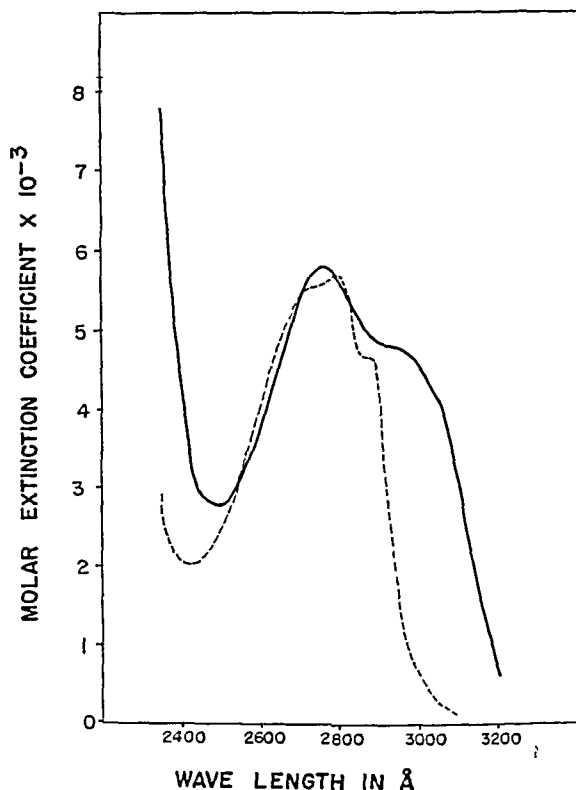


FIG. 1. Ultraviolet absorption spectra of serotonin (solid line) and tryptophan (dotted line) in water at pH 3.5.

point of inflection at 2930 Å, and a minimum at 2500 Å (Fig. 1). For purposes of comparison, the absorption spectrum of tryptophan (Eastman Kodak) in water at pH 3.6 is also presented. With respect to the location of maxima and points of inflection, neither of the curves shows significant change at pH 10.3.

*Potentiometric Titration*—21.5 mg. of serotonin were dissolved in 5.0 ml. of water and titrated with 0.0157 N carbonate-free sodium hydroxide at 26°. pH values were determined to the nearest 0.05 unit with a Beckman pH meter (glass electrode). Moles of acid or base combined per mole of

serotonin were calculated, assuming a molecular weight of 405 for serotonin. The experimental points are plotted in Fig. 2. The line is the theoretical dissociation curve for  $pK'_1$  of 4.9 and  $pK'_2$  of 9.8. The titration was not carried beyond pH 9.7 because of the inaccuracy of the electrode in this range and in order to prevent possible inactivation of the serotonin.

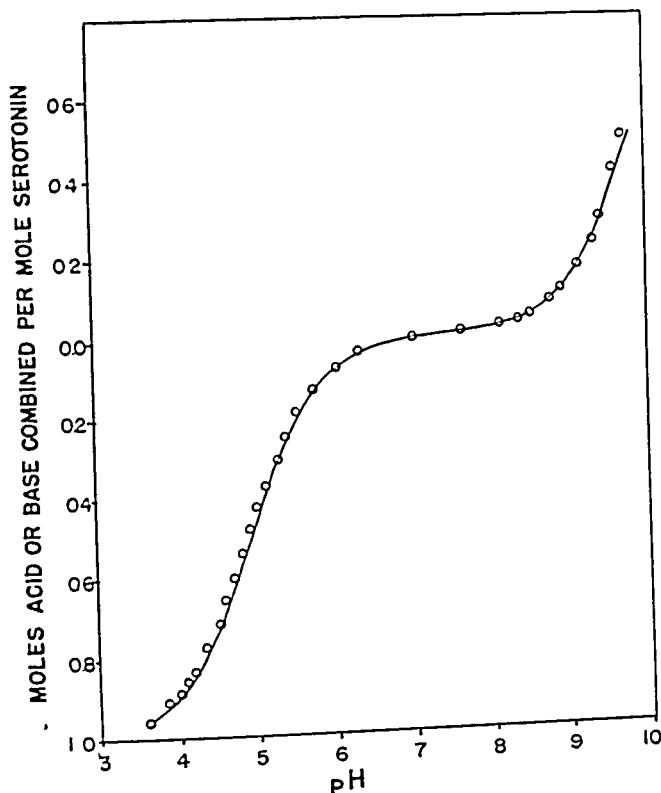


FIG. 2. Dissociation curve of serotonin. The points are experimental, the curve theoretical, based on the constants  $pK'_1 = 4.9$  and  $pK'_2 = 9.8$ .

Under identical conditions, 13.3 mg. of tryptophan were titrated. From the curve obtained,  $pK'_1$  of 2.4 and  $pK'_2$  of 9.4 were estimated, in good agreement with the published values of 2.38 and 9.39 (5).

*Color Reactions and Classification Tests*—Serotonin gives positive Hopkins-Cole, Ehrlich, Folin, and pine splinter tests.

In the Shaw-MacFarlane modification (6) of the Hopkins-Cole test with glyoxylic acid, 1 mole of serotonin gives a color equivalent to 0.89 mole of tryptophan. Visually, the color is violet-blue instead of the violet obtained with tryptophan. Without the glyoxylic acid, a pink color is developed by serotonin under these conditions.

In a modification<sup>5</sup> of the procedure of Graham *et al.* (7) for the Ehrlich *p*-dimethylaminobenzaldehyde reaction, 1 mole of serotonin gives a color equivalent to 1.10 moles of tryptophan. The final color is very similar for the two substances.

In the Folin-Ciocalteu reaction (8), 1 mole of serotonin gives a color equivalent to 2.4 moles of tryptophan.

The Sakaguchi reaction (9), the Bratton-Marshall method for diazotizable amines (10), the Gerngross-Voss-Herfeld reaction (11), and the Pauly diazo reaction for imidazoles (12) were all negative. In the latter two reactions, yellow colors were obtained containing no trace of red.

Qualitative ninhydrin, Molisch, and ferric chloride tests were negative. Serotonin heated with ninhydrin-sodium acetate produced a chrome yellow color.

The pine splinter test served the purpose of a rapid semiquantitative assay in the final stages of the isolation when carried out in the following way. 2 drops of concentrated hydrochloric acid were placed on a tongue depressor and spread with a glass slide. When the excess liquid had soaked into the wood, a trace of the sample to be tested was rubbed on the prepared wood surface. Fractions having an activity of 35,000 units or more per mg. gave a deep red color. The activity of fractions which still gave a detectable test was about 10,000 units per mg. (10 per cent pure).

Serotonin reduces ammoniacal silver nitrate, but the reaction is not a typical aldehyde response. In a test performed on 50  $\gamma$  of substance, a brown color formed within 1 minute, giving way to a red-brown precipitate in several hours, in turn followed by a black precipitate with slight mirror formation in 24 hours, the supernatant solution remaining reddish in color. With silver nitrate at pH 5, a slight reddish color and a black precipitate were observed after about 12 hours. The reaction was probably the same in both cases, occurring more rapidly under basic conditions.

With 10 per cent mercuric sulfate in 2.5 *N* sulfuric acid, serotonin, at a concentration of 1 mg. per ml., immediately forms a yellow precipitate which is very insoluble in water.

Despite the high percentage of nitrogen, it has not been possible to obtain a stable crystalline picrate with serotonin.

Solid serotonin catalyzes the decomposition of the iodine-azide complex in the Feigl test (13). In solution, however, at a concentration of 1 mg.

<sup>5</sup> To 0.5 ml. of the test solution, 1 drop of 2.5 per cent dimethylaminobenzaldehyde in 10 per cent  $H_2SO_4$ , 1 drop of 2 per cent  $NaNO_3$ , and 2.8 ml. of concentrated  $HCl$  are added. After standing at room temperature for 20 minutes, 7.0 ml. of 50 per cent (by volume) ethanol are added. The color is read with a No. 54 filter in the Klett-Summerson colorimeter.



per ml., the test is negative. A positive result is reportedly attributable to thio ketones and mercaptans; but since all the sulfur in serotonin appears to be present in the form of sulfate, an impurity may be responsible for the observed result.

Serotonin rapidly reacts with iodine in aqueous solution to give a water-insoluble compound. It does not give the typical tryptophan reaction with bromine in aqueous solution, although intensification of color has been observed in methanol solution.

#### DISCUSSION

The absorption spectrum and color reactions of serotonin are strongly indicative of the presence of an indole nucleus. This evidence, in combination with biogenetic considerations, suggests a structure which may be closely related to tryptophan or tryptamine. However, pronounced differences are apparent, and the calculated empirical formula focuses attention on two especially interesting aspects of structure; namely, the high nitrogen content and the high degree of saturation.

It is reasonable to assume that both of the groups revealed in the dissociation curve are basic in nature, since a sulfate salt is under consideration whose 0.01 M solution is at pH 3.6. Further, the absence of marked acidic properties, as revealed by lack of inactivation by diazomethane and non-adsorption on Amberlite IR-4B (14), supports this assumption. If, then, the two groups are basic, the stronger ( $pK'_b = 4.2$ ) approximates the base strength of trimethylamine and is what might be expected for a  $\beta$ -aryl ethylamine such as tryptamine, while the weaker ( $pK'_b = 9.1$ ) has the basicity of aromatic amines such as aniline ( $pK'_b = 9.4$ ) or N,N'-disubstituted guanidines such as creatinine ( $pK'_b = 9.2$ ). A third nitrogen can be accounted for in the indole nucleus. To speculate on the disposition of the remaining two nitrogens, they may be associated with the more weakly basic grouping in a disubstituted guanidine structure, or one or both may be combined in some grouping even less basic such as an amide. The formation of complexes with the amide type structure of dilituric acid and the ease of formation of ammonia on treatment with aqueous alkali<sup>6</sup> are consistent with these alternatives.

No primary amino groups are believed to be present in the molecule. The negative diazotizable amine test rules out *aromatic* primary amino groups, while the failure to form any red or violet coloration with ninhydrin-sodium acetate would seem to do the same for *aliphatic* primary amines.

The absence of quaternary nitrogen is indicated by the distillation, in a

<sup>6</sup> Unpublished experiments.

microsublimation apparatus, of a small sample of the free base, with apparent retention of full activity.<sup>7</sup>

Nothing is yet known of the disposition of the oxygen functions.

From the standpoint of chemical and pharmacological activity, the marked reducing power of serotonin, as evidenced by the reduction of gold, silver, and platinum salts, seems worth emphasizing. The results obtained by inactivation studies (14) would appear to be explained by this property in conjunction with the presence of the indole fragment. The reduction of the Folin reagent by serotonin to the extent of 2.4 times the reduction by tryptophan, mole for mole, indicates the presence of a second reducing group in addition to the indole nucleus. Epinephrine, the second most active constrictor substance in the perfused rabbit ear vessels, causes a mole for mole reduction of 3.0 times the reduction by tryptophan.

The similarity in chemical and biological activity of serotonin and epinephrine is apparent. The further *structural* similarity (one contains the indole ring system, the other is closely related to it) suggests the possibility that clarification of the structure of serotonin may reveal a more general relation between chemical structure and vasoconstrictor action than has been obtained by studying modifications of epinephrine structure.

We wish to acknowledge the valuable assistance of Miss Elizabeth Hunt and Mr. John Means, and to thank Dr. Hans Hirschmann of Western Reserve University for cooperation in determining the optical activity.

#### SUMMARY

Details of the method of isolating crystalline beef serum vasoconstrictor (serotonin) from a purified concentrate are presented. The color reactions and ultraviolet absorption spectrum indicate the presence of an indole nucleus in the structure. The structural implications of evidence based on color reactions and potentiometric titration are discussed.

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<sup>7</sup> Unpublished experiment. The oily distillate obtained (at 140°, 0.15 mm.) was dissolved in water. The retention of activity was judged by the correlation of the activity and the ultraviolet absorption spectrum of the solution.

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# SEPARATION AND DETERMINATION OF THE AMYLOSE AND AMYLOPECTIN FRACTIONS OF STARCH

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(Received for publication, July 14, 1948)

Most of the earlier procedures used for separating starch into amylose, the linear unbranched component, and amylopectin, the branched chain component (1, 2), have involved degradation and hydrolysis of the starch molecules. During the last few years, however, a number of methods have been proposed which fulfil, more or less, the necessary requirement of protecting the starch constituents from degradation. The more important of these methods for the separation of amylose from starch is based on its selective diffusibility in water at 60° or 80° (1, 3), precipitability with butanol (4), thymol (5), or nitroparaffins (6), and adsorbability on cellulose (7). It is shown in this communication that these methods fail to effect clear-cut as well as quantitative separation of the two starch fractions, while the purity of the products obtained is also variable. It has been possible, by suitable combination of certain of these procedures, to prepare amylose and amylopectin, judged for their purity by the intensity of their iodine colorations under standard conditions, and to determine their exact percentages in any starch preparation by reference to a calibrated curve for intensity of iodine coloration with known mixtures of the pure fractions (3).

## EXPERIMENTAL

*Preparation of Starch*—The major part of the studies reported here was carried out with a sample of starch prepared from a local variety of peas (*Pisum sativum*). The seeds, softened by soaking overnight in water, were ground to a not too fine consistency and the mash was extruded through a cloth bag into a sufficient volume of distilled water. The residual pulp was mashed and pressed out a second time. The combined extract was let stand and the sludge which separated was purified of proteinaceous material by repeated agitation and settling. The starch suspension was finally kneaded through muslin into water and centrifuged to separate the starch, which was washed successively with 20 and 80 per cent ethanol and allowed to dry at room temperature (28°). Analysis of the product gave 0.88 per cent protein, 0.25 per cent ether extractive, and 12.75 per cent moisture.

*Determination of Iodine Coloration*—The intensity of color developed in a 2 mg. per cent solution of starch or of the various starch fractions, on addition of a solution of iodine in potassium iodide to a final concentration of 4 mg. per cent of iodine, was measured in a 10 mm. cell by a Klett-Summerson photoelectric colorimeter with Filter K<sub>66</sub> in position (3). The colorimeter was initially adjusted so that the blank, which had a light yellow color due to the iodine in solution, gave a zero reading; the color measurements recorded are in terms of scale readings in the instrument.

*Fractionation of Starch by Selective Extraction of Amylose with Hot Water*—5 gm. of air-dry starch, mixed with water to avoid lump formation, were treated with about 300 ml. of water at 60° and the suspension maintained at this temperature for 4 hours with slow stirring. It was then centrifuged at 3000 R.P.M. and the supernatant passed through a sintered glass No. 4

TABLE I  
*Extraction of Starch with Water at 60°*

	On dry basis			Intensity of iodine color (scale readings)*		
	Yield 1	Yield 2	Yield 3	Yield 1	Yield 2	Yield 3
	per cent	per cent	per cent			
Original starch				143	149	148
Fraction I	14.4	14.7	15.8	279	271	269
“ II	0.9	0.6	Trace	181	180	
“ III	84.5	85.3	83.5	101	103	100

\* Klett-Summerson colorimeter.

filter, which was found more convenient to use than filter paper coated with Hyflo Super-Cel, as recommended in the original procedure (3). The clear filtrate, after addition of methanol to a concentration of 20 per cent volume per volume, was let stand for 48 hours. At the end of this period, the precipitated amylose (Fraction I) was filtered through a sintered glass No. 4 crucible, washed with 95 per cent ethanol, and finally with absolute alcohol before drying in a vacuum oven. The filtrate was further treated with methanol to 50 per cent volume per volume strength and allowed to settle as before. The precipitate (Fraction II) was filtered, washed, and dried to constant weight.

The gelatinous residue remaining after the centrifuging of the aqueous starch suspension was ground well, dehydrated by repeated additions of alcohol, filtered, and dried *in vacuo* (Fraction III). Table I gives a set of typical results obtained together with the iodine colorations of the starch and of the different fractions.

In the set of experiments given in Table II, the temperature of fractiona-

tion was kept at 80°, as recommended by Meyer (1); the procedure was otherwise the same as that described above.

Fractionations of pea starch and of the crude amylopectin (Fraction III, Table I) were also attempted by treatment for 48 hours at room temperature with 1:2 chloral hydrate solution in water, as recommended by Meyer. The products obtained gave iodine coloration averaging 74 and 67 respectively; by using chloral hydrate solution at 80°, the corresponding color readings were 54 and 36.

TABLE II  
*Fractionation of Starch with Water at 80°*

	Yield 1	Yield 2	Intensity of iodine color*	
			Yield 1	Yield 2
	<i>per cent</i>	<i>per cent</i>		
Fraction I . . . . .	16.0	16 3	220	224
" II . . . . .	Trace	Trace		
" III . . . . .	82 9	79 6	114	104

\* See Table I.

TABLE III  
*Fractionation by Butanol Extraction*

Fraction	Yield 1	Yield 2	Iodine coloration*	
			Yield 1	Yield 2
	<i>per cent</i>	<i>per cent</i>		
Butanol-pptd. by autoclaving	38 6	39 2	243	241
" " Waring blender	39 5	39 4	234	230
Butanol-non-pptd. by autoclaving	60 7	59 9	52	49
" " Waring blender	59.4	60 7	47	50

\* \* See Table I.

*Fractionation by Selective Precipitation of Amylose with Butanol*—Schoch's butanol precipitation method (4) was closely followed, except for the purification of the separated amylose by recrystallization from the boiling water-butanol mixture. Since it was thought that some hydrolytic degradation of starch was likely to occur as a result of the high temperature treatment under pressure, an attempt was made to eliminate autoclaving in Schoch's procedure by securing dispersion of starch with high speed stirring. A properly gelatinized paste of 5 gm. of starch in about 500 ml. of boiling water was treated in a Waring blender in two lots for 5 minutes each. Subsequent separation of the starch fractions was effected as described by Schoch. Typical results, by both procedures, are given in Table III.

*Fractional Precipitation of Amylose with Thymol*—The high speed stirring recommended by Haworth *et al.* (5) did not result in sufficient dispersion and it was found more effective to use a Waring blender for aiding dispersion. The results obtained by this method are shown in Table IV.

*Fractionation by Preferential Adsorption of Amylose on Cotton*—With a 1 per cent starch paste, gelatinized and dispersed in a Waring blender according to the procedure of Pacsu and Mullen (7), not more than 0.3 per cent of amylose (iodine coloration, average 223) was obtainable, therefore suggesting that preferential adsorption of amylose on the cotton used was far from satisfactory; somewhat similar results were obtained by using filter paper pulp as an adsorbent.

TABLE IV  
*Fractionation by Thymol Extraction*

Fraction	Yield 1	Yield 2	Yield 3	Iodine coloration*		
	per cent	per cent	per cent	Yield 1	Yield 2	Yield 3
Thymol-pptd.....	39.7	39.9	40.7	222	220	208
Thymol-non-pptd.....	60.1	60.2	59.1	50	52	45

\* See Table I.

#### DISCUSSION OF RESULTS

The pronounced difference in the affinity of amylose and amylopectin for iodine has formed the basis for the determination of their relative proportions in starches potentiometrically (8), absorptiometrically (3), or spectrophotometrically (9, 10). On the assumption that the intensity of iodine coloration with amylose or amylopectin fractions will be determined by their respective freedom from each other, it becomes apparent that the higher the scale reading, the purer will be the fraction in respect to its amylose content, and that, conversely, purity with regard to amylopectin will be connoted by the lowest scale reading. Based on this criterion, it would follow from the data presented here (Tables I to IV) that no single method effects simultaneously efficient and quantitative separation of the two starch fractions. The procedure of McCready and Hassid (Table I) gives, in one step, the purest amylose fraction, judging from its iodine-staining property; its solubility is, however, only of the order of 15 per cent, which is very low. That the amylopectin fraction obtained here is admixed with a high amount of amylose is evident from the values for iodine coloration as compared to the amylopectin fraction obtained by the procedures of Schoch (Table III) and of Haworth *et al.* (Table IV). The amylose fraction yielded by Meyer's extraction procedure (Table II) is only about 80 per cent as



pure as the corresponding fraction obtained according to the method of McCready and Hassid; this is no doubt due to the fractionation temperature being near the gelatinization point of the starch and consequent con-

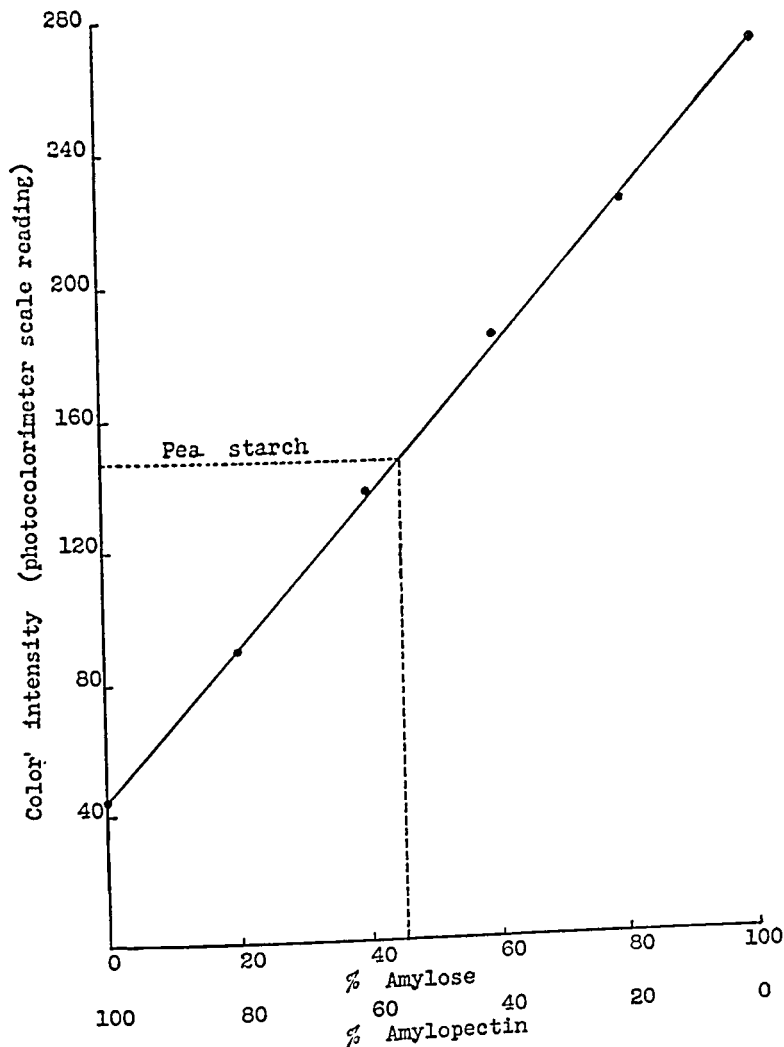


FIG. 1. Color intensities of mixtures of amylose and amylopectin from pea starch with iodine.

tamination with amylopectin by the disintegration and rupture of the granules. The use of chloral hydrate solution to purify amylopectin from admixed amylose resulted in a product still containing about 6 to 10 per cent of amylose.

Both butanol and thymol undoubtedly effect very much better fractionation of the starch components than does extraction with hot water, and, indeed, the yields of amylose and amylopectin correspond more nearly to the correct values deduced below than do those obtained by hot water extraction. However, it is clear from a comparison of the iodine coloration of amylose fractions (Tables III and IV) that they are respectively only about 85 and 77 per cent as pure as that obtained by the McCready and Hassid method. It has been possible to obtain pure amylose by successive recrystallizations from boiling water-butanol mixtures as recommended by Schoch (4, 11), but, the yields being no longer quantitative, it appeared preferable to do so in a single extraction with hot water at 60°.

Although the amylopectin fractions obtained by selective precipitation with butanol or thymol have given the lowest intensity of iodine coloration

TABLE V  
*Fractionation of Mung Starch*

Starch analysis	
Moisture, %.....	13.50
Proteins, %.....	0.69
Ether extractives, %.....	0.23
Iodine coloration of starch*.....	127
"      "      " amylose fraction*.....	287
"      "      " amylopectin fraction*.....	34
Amylose (from Fig. 2), %.....	36.8
Amylopectin (from Fig. 2), %.....	63.2

\* Scale readings, Klett-Summerson colorimeter.

of all the methods studied, it was felt that, since it is always the residue in the mother liquor after the amylose had been precipitated, amylopectin may not be easily obtainable in as pure a form as the amylose component. We therefore attempted to ascertain whether by butanol fractionation of the residue from the hot water treatment of starch at 60° (Fraction III, Table I) a purer preparation of amylopectin could be obtained than by Schoch's method from the original starch. By this procedure, a product was secured which gave an iodine coloration of only 43 or 44 units. This was the purest amylopectin obtainable; a product with similar purity could also be prepared by thymol fractionation of crude amylopectin.

Pure preparations of amylose and amylopectin can thus be obtained in one and two operations, respectively, by a combination of the features of McCready and Hassid's method for amylose and that of Schoch or of Haworth *et al.* for amylopectin. By using various proportions of the starch components prepared as above, the color intensities of the mixtures in

solution (2 mg. per 100 ml.) with iodine can be plotted against per cent concentration of the two fractions when a linear relationship similar to that reported by McCready and Hassid (3) is obtained (Fig. 1), and from which,

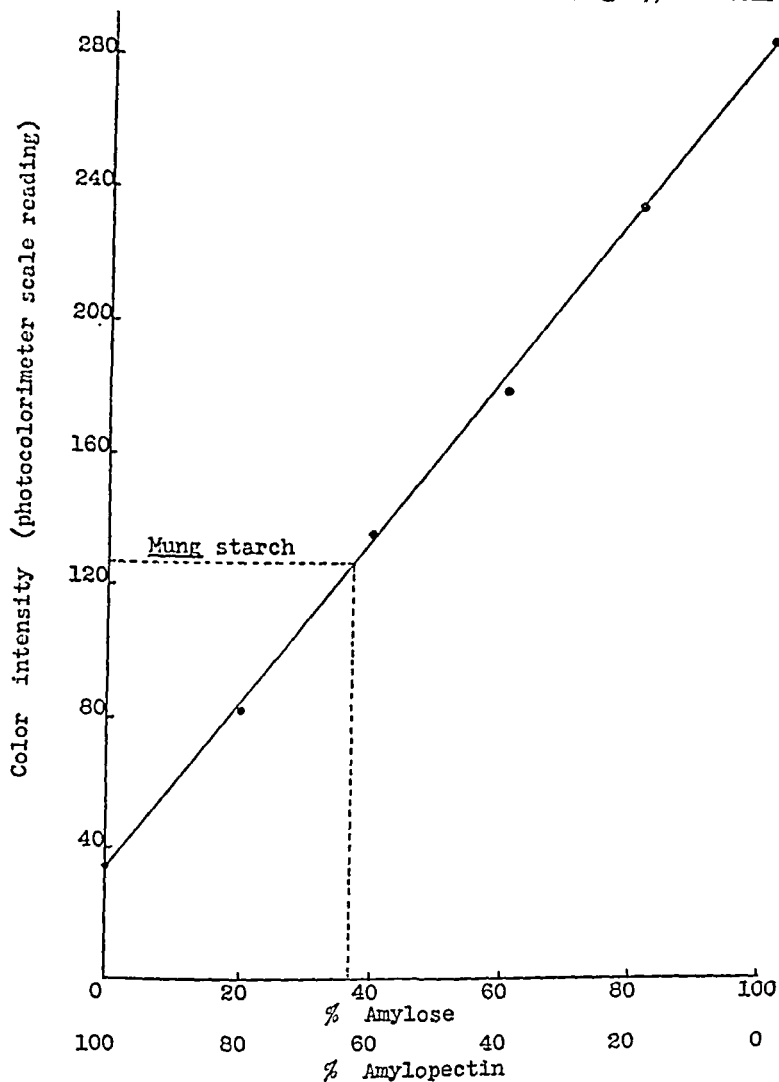


Fig. 2. Color intensities of mixtures of amylose and amylopectin from mung starch with iodine.

after ascertaining the color intensity of the original starch with iodine under identical conditions, its proportions of the two constituents can be read; the latter can also be deduced by simple extrapolation, as there is strict

proportionality between color intensity with iodine and amylose or amylopectin content. In this way, the preparation of pure starch used in these studies, with its iodine coloration of 147 (Table I), can be observed to consist of 45.0 per cent amylose and 55.0 per cent amylopectin.

By the foregoing procedures for the preparation of pure amylose and amylopectin fractions, and by using a preparation of mung (*Phaseolus radiatus*) starch, the resulting observations are given in Table V and in Fig. 2.

Differences such as are recorded here in intensities of iodine coloration with pure amylose or amylopectin preparations from natural starches are bound to exist because of possible heterogeneity as to molecular size as well as, with amylopectin, to variations in the degree of branching (cf. (8)).

Although the various methods for the fractionation of starch examined here do not effect a clear-cut and quantitative separation of the unbranched and branched components in their pure state, fractionation by selective precipitation of amylose with butanol or with thymol, as recommended by Schoch (4) and by Haworth *et al.* (5), gives an approximate idea of the relative proportions of the two constituents. However, to obtain them in a pure state for examination of their individual properties or for a precise evaluation of their percentages in any starch sample by reference to a calibrated curve or by extrapolation, as described here, it would appear necessary to resort to a combination of procedures involving the properties of amylose for selective diffusibility in water at 60° and precipitability with butanol- or thymol-saturated water.

#### SUMMARY

1. A comparative study has been made of the procedures for the fractionation of starch based on the differential solubilities of amylose and amylopectin in hot water and in butanol- or thymol-saturated water.

2. It is shown that the method of extraction with hot water at 60° yields an amylose fraction which is the purest obtainable, judged from the intensity of its coloration with iodine; amylose separation is not, however, quantitative.

3. Fractionation of starch by selective precipitation of amylose with butanol or thymol gives only a rough indication of the relative proportions of the linear and branched components; besides, separation, as judged by the iodine-staining properties of the products obtained, is not clear-cut.

4. A procedure is outlined for obtaining highly pure preparations of amylose and amylopectin from a starch sample. By quantitatively determining the color intensities of the starch and of known mixtures of its amylose and amylopectin fractions with iodine, their proportions in the former can be precisely estimated.

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# THE INCORPORATION OF ACETATE AND BUTYRATE CARBON INTO RAT LIVER GLYCOGEN BY PATHWAYS OTHER THAN CARBON DIOXIDE FIXATION\*

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(Received for publication, July 23, 1948)

This investigation was undertaken to study, by use of acetic and butyric acids labeled with  $C^{13}$ , the conversion in the intact animal of lower fatty acid carbon to liver glycogen in order to help explain the intermediate reactions linking fat and carbohydrate metabolism in the intact animal. It appears certain that such conversion does occur for both of these acids (1).

Classically, a dietary constituent has been considered a glycogen former if, after its administration, a net increase in glycogen is found over and above that of the control. By this criterion, there is no general agreement whether acetate and butyrate are glycogen formers. However, the carbon of a fatty acid could enter liver glycogen without effecting a net increase in the glycogen. For example, carbon could enter because of the constant turnover between carbohydrate and its precursors. Furthermore, because of the influence of the dynamic equilibrium between fats, proteins, and carbohydrates there could actually be a decrease in glycogen after administration of a fatty acid and still carbon of the fatty acid could enter the latter. Similarly, a net increase in glycogen could conceivably occur without transfer of carbon from the fatty acid to glycogen. The question of whether acetate and butyrate are liver glycogen formers in the classical sense is different from that being considered in the present work; namely, whether the carbon of these fatty acids is incorporated into liver glycogen.

The fatty acids in question are relatively rapidly metabolized to  $CO_2$  (1), and  $CO_2$  itself can be incorporated into liver glycogen (2). The biochemical significance of the presence of fatty acid carbon in glycogen therefore depends to a considerable degree upon (a) whether all such carbon entered glycogen via  $CO_2$  fixation or (b) whether some entered by another route without first being converted to  $CO_2$ .

It is possible to degrade the glucose units of liver glycogen to locate the

\* This work was supported in part by grants from the Graduate School of the University of Minnesota and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

position of the labeled carbon (2). In the case of  $\text{CO}_2$  fixation, the isotopic carbon has been detected only in positions 3 and 4 of the glucose (2).<sup>1</sup> Should the heavy carbon in glycogen isolated after feeding isotopic fatty acid appear in positions other than 3 and 4, this would be rather convincing evidence that a pathway other than  $\text{CO}_2$  fixation was involved. In the experiments to be reported, the distribution of  $\text{C}^{13}$  has been determined in rat liver glycogen after the administration, respectively, of each of the following acids:  $\text{CH}_3\text{C}^{13}\text{OOH}$ ,  $\text{C}^{13}\text{H}_3\text{COOH}$ ,  $\text{C}^{13}\text{H}_3\text{C}^{13}\text{OOH}$ ,  $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}^{13}\text{OOH}$ ,  $\text{CH}_3\text{CH}_2\text{C}^{13}\text{H}_2\text{COOH}$ ,  $\text{CH}_3\text{C}^{13}\text{H}_2\text{CH}_2\text{COOH}$ . Evidence has been obtained which indicates that these acids are converted to glycogen by another route in addition to  $\text{CO}_2$  fixation, and some indications have been obtained as to the mechanisms of these conversions. Preliminary reports of this work have been published previously (3-5).

### Methods

*Preparation of Isotopic Fatty Acids*—The carboxyl-labeled fatty acids were prepared by the Grignard reaction from the appropriate bromide and isotopic  $\text{CO}_2$ . Purity of these acids was checked by determination of titratable acidity, total carbon, and partition coefficients (6); all agreed with theoretical values except the partition coefficient of the  $\text{CH}_3\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{C}^{13}\text{OOH}$ , which was 30.6 as compared with 31.1 for known butyric acid.

The  $\text{C}^{13}\text{H}_3\text{COOH}$  was synthesized by a modification of the method of Anker (7). The purity of this and the following compounds was established by the same procedures that were used with the carboxyl-labeled acids. Agreement with theory was satisfactory unless otherwise indicated.

The  $\text{C}^{13}\text{H}_3\cdot\text{C}^{13}\text{OOH}$  was synthesized by a procedure which was based upon the method of Cramer and Kistiakowsky (8). In the modification 50 mm of  $\text{BaC}^{13}\text{O}_3$  were used. The acetylene-hydrogen mixture as formed on hydrolysis of the barium carbide magnesium was collected over water in a 3 liter flask and was then passed through a sintered glass disk into 150 ml. of boiling catalyzing solution. The resulting aldehyde was collected in 50 ml. of 5 per cent sodium bisulfite. The outgoing gas was collected and again passed through the catalyzing solution. The aldehyde was distilled from the bisulfite, after addition of calcium carbonate, and was oxidized to acetic acid by the dichromate method of Stahly *et al.* (9).

Butyric acid labeled in the  $\alpha$  position with  $\text{C}^{13}$  was prepared by the fol-

<sup>1</sup> In experiments carried out subsequently with  $\text{C}^{14}\text{O}_2$ , it has been found that actually a trace of isotope does appear in carbon atoms 1, 2, 5, and 6. The specific activity of these positions has been found to be 1 to 2 per cent of that of carbon atoms 3 and 4.



lowing series of reactions. Carboxyl-labeled sodium propionate was synthesized by the Grignard reaction and converted into the ethyl ester by heating with diethyl sulfate. The procedure was similar to the synthesis of carboxyl-labeled ethyl acetate (10). The ethyl propionate was then hydrogenated at 250° and 220 atmospheres by use of the copper-barium-chromium oxide catalyst (11, 12). The mixture of ethyl and propyl alcohols was converted into a mixture of the corresponding iodides by heating with constant boiling hydriodic acid (13). The iodides were separated by fractionation. This separation was not complete and there was some non-labeled ethyl iodide in the propyl iodide fraction. The isotopic propyl iodide was converted into  $\alpha$ -labeled butyric acid by the Grignard reaction. The over-all yield was 22 per cent of the theory. From 60 mm of isotopic  $\text{BaCO}_3$ , as determined by the partition coefficient, 13.1 mm of  $\alpha$ -labeled butyric acid were obtained, mixed with 5.3 mm of non-isotopic propionate.

Butyric acid labeled in the  $\beta$  position with  $\text{C}^{13}$  was synthesized by the following procedure. Carboxyl-labeled acetic acid was esterified with *n*-butyl alcohol and hydrogenated at 250° and 220 atmospheres with the copper-barium-chromium oxide catalyst. The mixture of ethyl and butyl alcohols was converted into the corresponding mixture of iodides and fractionated. The isotopic ethyl iodide was converted into butyric acid by the malonic ester procedure (14). The butyric acid and some non-labeled acetic acid from the malonic ester were converted into the methyl esters by heating the potassium salts with dimethyl sulfate and purified by fractionation. The methyl butyrate was saponified, and the butyric acid identified by determination of the partition coefficient. The over-all yield was 25 per cent. From 93.5 mm of sodium acetate 24.5 mm of  $\beta$ -labeled butyric acid mixed with 0.96 mm of non-isotopic acetic acid were obtained.

Before administration to the experimental animals, the solution of the sodium salt was made just acid to phenolphthalein by the addition of  $\text{HCl}$ .

*General Experimental Procedure*—The procedure of Buchanan *et al.* (1) has been adopted, in which glucose is fed to fasted rats, together with the isotopic fatty acid. Under such conditions a net deposition of isotope-containing glycogen occurs and there is relatively minor dilution of the newly formed glycogen by preformed non-isotopic glycogen.

Table I contains data concerning the animals used, the material administered, the liver weight, and the quantity of liver glycogen recovered.

Rats, 120 to 240 gm. in weight, raised on a diet of Promax rat food,<sup>2</sup> were fasted for approximately 24 hours. Immediately after their teeth were clipped, the rats were fed by stomach tube a solution containing 1.7 to 3.3

<sup>2</sup> Analysis by manufacturer, in per cent, protein 29.5, fat 8.40, fiber 2.8, nitrogen-free extract 41.45, moisture 9.00, ash 8.85, calcium 1.20, phosphorus 1.13.

mm of fatty acid and 353 to 477 mg. of glucose per 100 gm. of body weight. The total volume administered was usually such as to make the glucose concentration 20 per cent.

TABLE I  
General Experimental Data

Rat No.	Weight after fast	Sex	Duration of fast	Fatty acid administered	Amount administered	Glucose administered	Liver weight	Liver glycogen (as glucose)
	gm.		hrs.		mm per 100 gm. body weight	mg. per 100 gm. body weight	gm.	mg.
33	234	♂	24	CH <sub>3</sub> C <sup>13</sup> OOH	2.5	400	8.4	29
34	215	♂	24	"	2.5	400	7.6	
38	215	♀	50	"	2.5	400	5.8	
100	155	♀	24	C <sup>13</sup> H <sub>3</sub> COOH	2.4	390	5.5	188
101	148	♀	24	"	2.4	400	5.8	
102	153	♀	24	"	2.4	400	5.8	
103	165	♀	24	"	2.5	400	5.5	116
51	190	♂	24	C <sup>13</sup> H <sub>3</sub> C <sup>13</sup> OOH	2.9	420	5.6	
63	210	♂	27	"	2.7	410	7.5	
64	210	♂	27	"	2.7	410	7.4	85
69	200	♀	24	"	2.8	450	5.9	
70	200	♀	24	"	2.8	450	5.2	
71	183	♀	24	"	2.8	438	6.7	500
72	179	♀	24	"	2.8	438	6.3	
73	125	♂	24	"	2.3	400	4.9	
74	135	♂	24	"	2.3	400	5.5	115
75	140	♂	24	"	2.3	400	5.5	
53	240	♂	24	CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> C <sup>13</sup> OOH	2.6	417	8.3	
67	230	♂	24	"	2.5	400	6.7	90
81	140	♂	24	CH <sub>3</sub> CH <sub>2</sub> C <sup>13</sup> H <sub>2</sub> COOH	1.82*	408	6.3	140
82	155	♂	24	"	1.79*	402	6.2	
83	160	♂	24	"	1.81*	406	6.6	
84	140	♂	24	"	1.74*	390	6.1	378
89	140	♂	24	CH <sub>3</sub> C <sup>13</sup> H <sub>2</sub> CH <sub>2</sub> COOH	2.5	400	6.1	
90	130	♂	24	"	2.5	400	6.2	
91	130	♂	24	"	2.5	400	5.5	91
92	130	♂	24	"	2.5	400	5.8	105

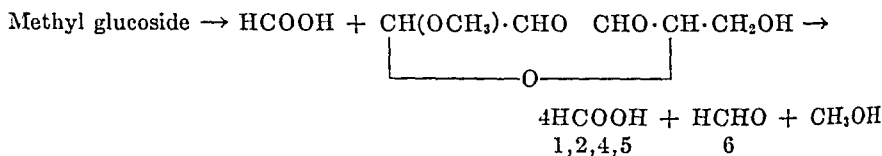
\* The solution contained appreciable amounts of non-isotopic propionate.

The animals were placed in a metabolism chamber for collection of their respiratory CO<sub>2</sub> in NaOH, as previously described (2), with the modification that in the experiments other than those with carboxyl-labeled acids both the alkali and air were stirred. There were three collection periods of 50 minutes each, between which the chamber was opened for changing



1, 2, 5, and 6 were obtained in one fraction. Ether extraction of the lactic acid was performed only in experiments on Rats 69 to 75 and Rats 100 to 103; in other instances the oxidation was carried out directly on the centrifuged solution from the bacterial fermentation.

In the chemical degradation, with limitations as described previously (2) and in this communication, carbon atoms 3 and 6 are obtained as individual fractions and carbon atoms 1, 2, 4, and 5 as a third fraction.



Heavy carbon was determined with the mass spectrometer. The values are expressed as atom per cent excess  $\text{C}^{13}$ , *i.e.*  $\text{C}^{13}$  in excess of normal (un-enriched) carbon, commercial reagent grade  $\text{BaCO}_3$  being employed as a source of a  $\text{CO}_2$  standard in calibrating the mass spectrometer. The maximum difference in  $\text{C}^{13}$  content between this standard and normal rat liver glycogen was found to be 0.005 atom per cent. Whereas this result might indicate 0.005 to be the expected experimental variation, it has been our procedure not to place significance on values less than 0.02 atom per cent in excess of normal. Thus a margin is provided for possible incomplete separation of the isotopic and non-isotopic compounds.

In a control experiment non-isotopic acetate was administered to the animal, but at the time the liver was placed in  $\text{KOH}$ , carboxyl-labeled acetate was also added to the alkali. Significant amounts of excess  $\text{C}^{13}$  were not found in the glycogen (see Table II, results for Rat 35).

#### RESULTS AND DISCUSSION

The discussion will take the following order. First the data on the relative distribution of the isotope in the glucose unit of the glycogen will be considered for each of the different types of labeled acids. Next evidence will be presented which shows that these acids are converted to glycogen by a pathway in addition to that of  $\text{CO}_2$  fixation. Finally the intermediary mechanism of metabolism of acetate and butyrate will be considered in relation to the tricarboxylic acid cycle and glycogen synthesis.

##### *Distribution of Isotope in Glucose from Liver Glycogen*

The distribution of  $\text{C}^{13}$  in the liver glycogen following the administration of labeled fatty acids is shown in Table II; the values are from both the bacterial and chemical degradations. Although there are some discrepancies between results by the two methods, which will be considered later,

TABLE II  
Distribution of  $C^{13}$  in Liver Glycogen after Administration of Labeled Fatty Acids  
 $C^{13}$  values are expressed in terms of atom per cent excess.

Fatty acid administered	$C^{13}$ whole fatty acid molecule	Rat No.	$C^{13}$ in bacterial degradation fractions				$C^{13}$ in chemical degradation fractions				$C^{13}$ whole glucose molecule	Location of isotope in glycogen
			Carbon atoms of glucose				Carbon atoms of glucose					
			3,4	2,5	1,6		3	1,2,4,5	6			
$CH_3C^{12}OOH$	0.00	35	0.015	0.01							$C-C-C-C-C-C$	
$CH_3C^{13}OOH$	2.63	33, 34	0.10	0.01							$C-C-C^*-C^*-C-C$	
$C^{13}H_3COOH$	1.98	100-103	0.08	0.18	0.16†		0.10	0.17	0.19	0.14	$C^*-C^*-C^0-C^0-C^*-C^*$	
$C^{13}H_2C^{13}OOH$	4.30	51	0.24	0.19	0.14†						$C^*-C^*-C^*-C^*-C^*-C^*$	
"	4.30	63, 64					0.29	0.25	0.23	0.26	$C^*-C^*-C^*-C^*-C^*-C^*$	
"	4.60	69-75	0.36	0.29	0.28§		0.32	0.28	0.24	0.29	$C-C-C^*-C^*-C-C$	
$CH_3CH_2CH_2C^{13}OOH$	0.98	53	0.13	0.01	0.01						$C-C-C^*-C^*-C-C$	
"	0.98	67	0.18	0.02	0.01						$C-C-C^*-C^*-C-C$	
$CH_3CH_2C^{13}H_2COOH$	0.97	81	0.04	0.17	0.14						$C^*-C^*-C^0-C^0-C^*-C^*$	
"	0.97	82-84	0.05	0.16	0.14¶		0.05	0.14	0.17	0.14	$C^*-C^*-C^0-C^0-C^*-C^*$	
"	1.09	90	0.12	0.02	0.01						$C-C-C^*-C^*-C-C$	
$CH_3C^{13}H_2CH_2COOH$	1.09	89, 91, 92	0.16	0.02	0.02		0.11	0.05	0.01	0.06	$C-C-C^*-C^*-C-C$	

Mg. of  $CO_2$  in sample (†) 28.5, (‡) 8.2, (§) 19.8, (||) 15.0, (¶) 14.9.

the over-all results can be summarized from the data as given. It is seen that with both carboxyl-labeled acetic and butyric acids glucose was obtained in which the isotope was predominantly in the 3 and 4 positions. There was little or no excess in the 2,5 and 1,6 positions. This fact has been indicated in the last column of Table II by means of a 6-carbon skeleton ( $C-C-C^*-C^*-C-C$ ) in which the asterisk locates the preponderance of isotope. A similar glucose is obtained with the  $\beta$ -labeled butyrate. On the other hand, with methyl-labeled and doubly labeled acetate and with  $\alpha$ -labeled butyrate, a significant concentration of isotope was found in all degradation fractions and the indications are that each position of the glucose contained isotope. In the case of the methyl-labeled acetate and  $\alpha$ -labeled butyrate the 3 and 4 positions contained a *smaller* but significant excess of isotope than the other positions, and this type of glucose has been indicated as follows:  $C^*-C^*-C^0-C^0-C^*-C^*$ . With the doubly labeled acetate all positions were high, but the 3 and 4 positions were the highest.

Before further consideration is given to the significance of these data, the reliability of the degradation procedures will be discussed. It will be noted in Table II that whenever there was an excess of isotope in the 2,5 and 1,6 positions as obtained by the bacterial degradation the concentrations were usually of the same order of magnitude in the two fractions, but frequently the 1 and 6 positions contained somewhat less excess  $C^{13}$  than did the 2 and 5 positions. It was of considerable importance to establish whether or not these small differences were true differences, for, as will be shown in the later discussion, an unequal concentration would not be compatible with the currently accepted schemes of carbohydrate metabolism.

It has been found that the observed values are not true ones and that an error is caused by traces of extraneous carbon which are oxidized to  $CO_2$  by the chromic acid oxidation used to convert the iodoform carbon to  $CO_2$ . Dilution from this source may be appreciable when samples of the size usually dealt with in the present work are involved (0.5 mm of  $CO_2$  and less). In the case of the 2,5 position when mercuric acetate is used to convert the formic acid to  $CO_2$ , no comparable dilution occurs.

Some of the evidence on this point follows: When  $CO_2$  was liberated from  $BaC^{13}O_3$  by the chromic acid oxidant, it regularly displayed a lower  $C^{13}$  concentration than the original  $BaC^{13}O_3$ , unless enough  $BaC^{13}O_3$  was used to obviate the effects of dilution by the extraneous carbon which was oxidized to  $CO_2$ . The same type of dilution was observed when the chromic acid oxidation was applied to a labeled organic compound ( $C^{13}H_5CH_2COOH$ ). The resulting  $CO_2$  contained a progressively greater  $C^{13}$  concentration as the sample size was increased (from 5 to 40 mg. of  $CO_2$ ). When lactic acid, which was synthesized chemically (8) from isotopic acetylene, and thus contained an equal concentration of  $C^{13}$  in the  $\alpha$ - and  $\beta$ -carbons,

was degraded in the usual way, a discrepancy was noted between the labeled positions similar to that found with the lactate from liver glycogen. With a large enough sample the  $C^{13}$  content of the  $CO_2$  derived from the  $\alpha$ - and  $\beta$ -carbons of the synthetic lactate was found to be the same. These results are summarized in Table III. In addition when two aliquots of the lactate from the liver glycogen of Rats 69 to 75 were degraded, the smaller samples showed a discrepancy between the  $C^{13}$  content of the  $\alpha$ - and  $\beta$ -carbons, whereas the larger samples contained practically identical isotope concentrations (Table III). It is thus clear that with small samples false and variably low values were obtained for the 1 and 6 positions.

The results indicate with reasonable certainty that there is no difference between the 2,5 and 1,6 positions, and that any difference shown in Table

TABLE III

*Effect of Sample Size on Apparent Isotope Content of  $\alpha$ - and  $\beta$ -Carbon Atoms of Labeled Lactate*

Material degraded	$\alpha$ -Carbon		$\beta$ -Carbon	
	$CO_2$	$C^{13}$	$CO_2$	$C^{13}$
	mg.	atom per cent excess	mg.	atom per cent excess
Synthetic $C^{13}H_3C^{13}HOHCOOH$ .	10.6	3.32	10.6	2.62
" " "	21.1	3.37	18.1	2.96
" " *	24.9	1.14	31.5	1.16
Aliquot of lactate from Rats 69-75	8.3	0.28	12.5	0.22
" " " " " 69-75 ....	18.2	0.29	19.8	0.28

\* Run on a sample of lactate containing less isotope than that used for the first two degradations.

II may be the result of the small samples employed. The present results are therefore considered to be in agreement with the idea that the 2,5 and 1,6 positions contained equal concentrations of isotope.

It should be pointed out that the degradation cleanly separates the different positions of the lactic acid. Thus in the degradation of synthetic  $C^{13}H_3C^{13}HOHCOOH$ , the  $CO_2$  isolated from the carboxyl group contained no excess  $C^{13}$  and the  $\alpha$ - and  $\beta$ -carbon fractions contained equal  $C^{13}$  concentrations. On the other hand when a sample of synthetic  $CH_3C^{13}HOHCOOH$  was degraded, it yielded excess isotope only in the single fraction corresponding to the  $\alpha$ -carbon atom.

The results from the chemical degradation will be considered next. The data from this degradation in conjunction with the data from the bacterial degradation provide an opportunity to determine in greater detail the distribution of the isotope in the sugar.

Sufficient material was available in four of the glucose samples for both a bacterial and a chemical degradation. In the chemical degradation, probably the most reliable result is for position 6, since the formation of formaldehyde in the periodic acid oxidation is quite specific for primary alcohols. It will be noted in Table II that position 6 as determined by chemical degradation contained approximately the same concentration of isotope as did the 1,6 and 2,5 fractions obtained from bacterial degradation. The only exception is the result from the doubly labeled acetate, Rats 69 to 75, in which the concentration of isotope in position 6 was 0.24 as compared to that of 0.29 and 0.28 for the 2,5 and 1,6 positions. Possibly this result may be in error. A discrepancy in the results is indicated, because the average concentration of all fractions by bacterial degradation was higher than those from chemical degradation.

On the whole, however, it is seen that the results are in agreement with the idea that carbon atoms 1, 2, 5, and 6 contain an equal concentration of isotope. This follows from the observation that in general carbon atom  $6 = 1,6$ ; therefore carbon atom  $1 = 6$ ; also  $1$  or  $6$  or  $1,6 = 2,5$ . It is to be noted that, although the results offer direct data indicating equality of positions 1 and 6, they do not give direct evidence that position 2 equals position 5. It seems likely, however, that this is the case from a consideration of the schemes of glycolysis.

An evaluation of the relative concentration of  $C^{13}$  in positions 3 and 4 may be obtained from the value for position 3 as determined chemically and that of the value for combined positions 3 and 4 from bacterial degradation. Only a rough approximation is possible, however, because position 3 in the chemical degradation is not completely specific. Position 3 as obtained in this method consists of formic acid and it is contaminated by formic acid arising from other positions. In part this contamination comes from unmethylated glucose, since the samples of glucoside contained at least 1 to 2 per cent free glucose. In addition some free glucose may be formed by hydrolysis of the glucoside during the oxidation. In the periodic acid oxidation of free glucose formic acid is formed from 5 of the 6 carbon atoms. The fact that contamination does occur is indicated by results obtained on glucoside prepared from pure glucose in which more than a mole for mole yield of formic acid was found (2). Contamination of position 3 becomes apparent when the 1,6 and 2,5 positions have a low or high concentration of isotope as compared to the 3 and 4 positions. Thus when 1, 2, 5, and 6 carbon atoms contained more isotope than carbons 3 and 4, the contamination gave a high value for carbon atom 3 as compared to carbons 3 and 4; on the other hand when carbon atoms 1, 2, 5, and 6 contained a low concentration of isotope, the opposite effect occurred. These results were noted in most cases (see Table II).

When consideration is taken of these sources of contamination of posi-



tion 3, comparison of position 3 from chemical degradation with positions 3 and 4 from bacterial degradation indicates that position 3 is approximately equal to positions 3 and 4. Thus carbon atoms 3 and 4 probably contain an equal concentration of  $C^{13}$ . All the results of Table II, therefore, are in conformity with the idea that the two halves of glucose are identical. This is the distribution of isotope which is to be expected on the basis of the conventional schemes of glycolysis in which glucose is formed on an over-all basis by union of 2 pyruvate molecules through a carboxyl to carboxyl linkage (2). This prediction assumes that the triose isomerase acts sufficiently rapidly to equilibrate the isotope in the glyceraldehyde phosphate and dihydroxyacetone phosphate. It is apparent, however, that the methods do not permit the strictly quantitative measurement which would be necessary to prove this point rigidly.

*Evidence for Conversion of Fatty Acid Carbon to Glycogen by Pathway  
Other Than  $CO_2$  Fixation*

It is apparent from Table II that after the feeding of carboxyl-labeled acetate or butyrate, as well as of  $\beta$ -labeled butyrate, all, or practically all, of the detectable excess  $C^{13}$  was found in positions 3 and 4 of the glucose. Since these are the same positions as for  $CO_2$  fixation, the results with these compounds provide no direct evidence that there is a mechanism other than  $CO_2$  fixation by which the carbon of acetic or butyric acid is incorporated into liver glycogen. However, after the feeding of  $\alpha$ -labeled acetate, doubly labeled acetate, or of  $\alpha$ -labeled butyrate, not only carbon atoms 3 and 4, but all degradation fractions contained significant amounts of  $C^{13}$ . These latter results furnish direct evidence that at least the  $\alpha$ -carbon atoms of the fatty acids studied are incorporated into liver glycogen by some means in addition to  $CO_2$  fixation. The possibility that the presence of fatty acid has provided a route over which carbon may be incorporated as  $CO_2$  into positions other than 3 and 4 of the glucose is excluded by the results obtained when the carboxyl-labeled acids were fed.

The level of isotope in the respiratory  $CO_2$  has been used in attempting to evaluate the extent to which isotope from administered fatty acids reaches liver glycogen by  $CO_2$  fixation. Buchanan *et al.* (1) fed rats acetate, propionate, and butyrate labeled with  $C^{11}$  in the carboxyl position. On the basis of the radioactivity found in the liver glycogen and respiratory  $CO_2$  in these experiments as compared with the radioactivity found in liver glycogen and respiratory  $CO_2$  in  $CO_2$  fixation experiments, they attempted to apply a correction to the fatty acid experiments for the isotope deposited in the liver glycogen from  $CO_2$ . An observed isotope content in the glycogen over and above that calculated as derived from  $CO_2$  was taken to indicate conversion of fatty acid to glycogen by a second mechanism. These workers concluded that in the case of acetate  $CO_2$  fixation alone could ac-

count for all of the  $C^{11}$  of the glycogen, whereas for propionate and butyrate  $CO_2$  fixation could account for only a fraction of the isotope found.

In the present work, in the case of acetate as well as of butyrate, for all types of labeling employed, the isotope was more abundant in carbon atoms 3 and 4 of the glucose than would be anticipated from the level of  $C^{13}$  in the respiratory  $CO_2$  were  $CO_2$  fixation the only mechanism at work. For example, the excess  $C^{13}$  in carbon atoms 3 and 4 of the glucose isolated from the liver of rats administered  $NaHC^{13}O_3$  was only about one-sixth to one-seventh that found in the respiratory  $CO_2$  (2), while in the experiments with carboxyl-labeled acetate, the isotope in positions 3 and 4 was relatively twice as abundant as in the  $CO_2$  fixation experiments, being about one-third the excess noted in the respiratory  $CO_2$ .

Certain objections to comparisons of this sort may be pointed out. Experiments on  $CO_2$  fixation in which labeled bicarbonate has been given may not be regarded as suitable controls for experiments in which fatty acids have been fed, since the influence of fatty acid feeding on  $CO_2$  fixation has not been assessed. Furthermore, the isotope content of the respiratory  $CO_2$  may not bear the same relationship to the isotope content of the intracellular  $CO_2$  in both instances, since the isotopic  $CO_2$  has been introduced into the animal as bicarbonate in one case and has arisen from the intracellular oxidation of an organic acid in the other. The experiments of Ball *et al.* (21), in which  $NaHC^{11}O_3$  was administered and the specific activity of the  $NaHCO_2$  of pancreatic juice and of the respiratory  $CO_2$  was found to be the same, cannot be considered as establishing the respiratory  $CO_2$  as a valid sample of the intracellular  $CO_2$  of all tissue cells with respect to isotope content, since the cells of external secretion of the pancreas are probably unique in their ability to concentrate bicarbonate. Hence it is apparent that experiments with the carboxyl-labeled acids provide only indirect evidence regarding the transfer of the fatty acid carbon to glycogen by ways other than  $CO_2$  fixation, and, in view of the possible source of error described above, it is not surprising that the evidence of Buchanan *et al.* (1) and of the present work is in conflict with respect to acetate.

The present results with  $\alpha$ -labeled and doubly labeled acetate, in which the isotope occurs in positions other than 3 and 4 of liver glucose, show conclusively, however, that this acid is converted to carbohydrate by pathways other than  $CO_2$  fixation.

#### *Intermediary Metabolic Steps in Conversion of Fatty Acid to Glycogen*

Although in the present work we are observing only the initial and final stages whereby fatty acid carbon is transformed to glycogen, some discussion seems desirable concerning intermediate steps.



Because of the impressive evidence accumulating in favor of the tricarboxylic acid, or Krebs cycle, as a general mechanism for the oxidation of carbohydrates, fats, and proteins, the present results will be considered in terms of this cycle. The cycle, abridged for purposes of simplicity, is presented schematically in the accompanying diagram. The assumptions are made that the constituent hexose units of glycogen are formed from two 3-carbon fragments (represented by pyruvate) in accordance with the conventional schemes of glycolysis, and that the tricarboxylic acid cycle participates in the introduction of isotope into the 3-carbon fragment, pyruvate.

It must be emphasized that the following discussion of metabolic pathways for acetic and butyric acids is speculative. The possibility exists that the results of the present experiments, as well as of those on  $\text{CO}_2$  fixation (2), may be explained by unknown metabolic conversions, or by modifications of reactions in the tricarboxylic acid cycle, or by reactions on the path between pyruvate and glucose.

In the diagram it is to be noted that the carboxyl carbon of pyruvate becomes the 3 and 4 carbons of the glucose, the  $\alpha$ -carbon becomes the 2 and 5 carbons, and the  $\beta$ -carbon becomes the 1 and 6 carbons (Reaction a). Furthermore, since pyruvate is formed from the oxalacetate (Reaction b) by loss of the carboxyl adjacent to the methylene group ( $\beta$ -carboxyl group), the location of  $\text{C}^{13}$  in the oxalacetate determines the location of the isotope in the pyruvate.

The oxalacetate formed from pyruvate and isotopic carbon dioxide would contain labeled carbon in the  $\beta$ -carboxyl group only. In order to explain the location of fixed carbon dioxide in positions 3 and 4 of glycogen, it is assumed that the oxalacetate is converted to a symmetrical  $\text{C}_4$ -dicarboxylic acid, represented by succinic acid in the scheme (Reaction c). In this way the labeled carbon becomes randomized between both carboxyl groups. Reversal of Reactions c and b would then yield carboxyl-labeled pyruvate and hence glycogen labeled in positions 3 and 4.

*Pathways from Acetate to Glycogen*—From carboxyl-labeled acetate the isotope is transferred via the cycle to both carboxyl groups of the oxalacetate (Reactions d, e, f, g, c) and decarboxylation of the oxalacetate results in carboxyl-labeled pyruvate (Reaction b). Moreover, reentry of this labeled oxalacetate into the cycle cannot introduce the  $\text{C}^{13}$  into any other position in the oxalacetate, because both carboxyl groups of the oxalacetate are lost as  $\text{CO}_2$  in the transformations of the cycle. This condition also applies in the case of  $\text{CO}_2$  fixation. The scheme is thus in agreement with the observed results of Table II in that it indicates that either fixed carbon dioxide or the carboxyl of acetate will be only in the 3 and 4 positions of the glucose.

The results following the feeding of  $\alpha$ -labeled acetate are also explained.

Condensation of  $C^{13}H_3COOH$  with oxalacetate would lead through the cycle to the formation of oxalacetate, the non-carboxyl carbons of which are labeled. Decarboxylation of such oxalacetate yields  $\alpha, \beta$ -labeled pyruvate and, hence, glycogen labeled equally in carbon atoms 1, 2, 5, and 6. These carbon atoms of the glycogen were found to contain excess isotope, but it will be observed in Table II that carbon atoms 3 and 4 also contained an excess of  $C^{13}$ , up to nearly one-half that in the other positions. Part of this labeled carbon undoubtedly was introduced by reentry into the cycle of isotopic oxalacetate generated in the cycle. Assuming recirculation in the cycle of such oxalacetate, one finds upon study of the cycle that at the end of the second circulation the  $C^{13}$  concentration in the carboxyl groups of this compound is one-third that of the non-carboxyl carbons, and that with further recirculation of the oxalacetate the ratio of the isotope concentration in the carboxyl carbons to that in the non-carboxyl carbons approaches one-half. The methyl carbon of acetate can thus contribute significantly to the 3 and 4 positions of the glycogen, without conversion to  $CO_2$ .

The condensation of doubly labeled acetate with oxalacetate would lead through the cycle to the formation of oxalacetate, all carbon atoms of which are labeled; decarboxylation of such oxalacetate yields pyruvate and hence glycogen likewise with all its carbons labeled. Again the experimental results are in agreement with this expectation. If the route described is the only one by which acetate carbon is being converted to glycogen, the isotope concentrations should be equal in each carbon atom of the glucose chain. The deviation of experimental observations from this anticipation, *i.e.* a greater abundance of isotope in carbon atoms 3 and 4 than in the other fractions of the glycogen, is reasonably explained by the introduction of extra  $C^{13}$  into these positions by  $CO_2$  fixation. The fact that the observed  $C^{13}$  content of the respiratory  $CO_2$  (Table IV) was high in the experiments with the doubly labeled acetate is consistent with the idea that the extra  $C^{13}$  came from  $CO_2$ . The high  $C^{13}$  content in the respiratory  $CO_2$  resulted because both carbons of the acid were labeled and therefore the average excess  $C^{13}$  content was higher in this case than for the other acids administered.

In addition to comparing the agreement between the predictions of the scheme and the observed data relative to the distribution of the isotope in the carbon chain of the glucose unit, there is also opportunity for a similar comparison on the basis of recovery of the isotope in the respiratory  $CO_2$ .

The data on the respiratory  $CO_2$  are shown in Table IV. The total respiratory  $CO_2$  (column (a)) represents the sum of values for the three collection periods during which approximately equal quantities of  $CO_2$  were obtained in each period. The total  $C^{13}$  shown in column (b) was obtained

by summation of calculated  $C^{13}$  values for these three periods. In column (c) is shown the per cent of the administered  $C^{13}$  recovered in the respiratory  $CO_2$ . These values were calculated from column (b) and the mm of  $C^{13}$  administered, which may be calculated from the per cent  $C^{13}$  in

TABLE IV  
*Data on Respiratory  $CO_2$*

Fatty acid administered	Rat No.	$C^{13}$ of respiratory $CO_2$ by collection periods			Total respiratory $CO_2$	Total $C^{13}$ in respiratory $CO_2$	Administered $C^{13}$ recovered in respiratory $CO_2$	Respiratory $CO_2$ from administered fatty acid
		1	2	3	(a)	(b)	(c)	(d)
		atom per cent excess	atom per cent excess	atom per cent excess	mm	mm	per cent	per cent
$CH_3C^{13}OOH$	33, 34	0.20	0.33	0.37	37.4	0.115	19 (24)*	12
"	38	0.20	0.44	0.39	11.2	0.047	16 (20)*	16
$C^{13}H_3COOH$	100, 101	0.16	0.35	0.32	37.1	0.100	35	14
"	102, 103	0.20	0.31	0.32	35.4	0.097	31	14
$C^{13}H_3C^{13}OOH$	51	0.34	0.60	0.59	18.3	0.094	20	12
"	63	0.41	0.66	0.61	20.8	0.119	24	13
"	64	0.42	0.64	0.61	22.2	0.127	26	13
"	69	0.41	0.69	0.74	18.6	0.114	22	13
"	70	0.70	0.90	0.62	19.5	0.137	27	15
"	71	0.54	0.95	0.91	19.4	0.158	34	18
"	72	0.50	0.76	0.72	20.1	0.134	29	14
"	73, 74	0.39	0.58	0.65	31.5	0.173	30	12
$CH_3CH_2CH_2C^{13}OOH$	53	0.17	0.27	0.19	23.1	0.050	20	22
"	67	0.20	0.36	0.33	20.0	0.061	27	31
$CH_3CH_2C^{13}H_2COOH$	81, 82	0.07	0.11	0.14	32.9	0.035	17	11
"	83, 84	0.06	(0.10)†	0.13	30.1	0.028	14	10
$CH_3C^{13}H_2CH_2COOH$	89, 90	0.26	0.31	0.26	31.1	0.086	29	26
"	91, 92	0.28	0.35	0.29	33.5	0.103	36	28

\* The collection periods were 40 minutes each. The values in parentheses are the observed values multiplied by 5/4 to make the results comparable with the rest of the experiments.

† Assumed value.

the administered acid (Table II) and the amount of fatty acid given (Table I).

The values in column (c) indicate the proportion of the total amount of the isotopic carbon of administered acetate which was oxidized to  $CO_2$  in  $2\frac{1}{2}$  hours under the conditions of the experiment. The values in column (d) present a different aspect in that they indicate what part of the respiratory  $CO_2$  came from the fatty acid compared with that from other non-

labeled materials in the metabolic pool of the animal. The figures in column (d) indicate simply the dilution of the administered isotope by non-isotopic carbon and were obtained by the following calculation.

$$\frac{\text{Atom } \% \text{ excess } C^{13} \text{ in respiratory } CO_2}{\text{Atom } \% \text{ excess } C^{13} \text{ in fed fatty acid}} \times 100$$

The values for the denominator are given in Table II, while those for the numerator may be calculated from Table IV, columns (a) and (b).

It should be emphasized that this calculation (column (d)) assumes (except for the doubly labeled acid, for which no such assumption is necessary since both carbon atoms contain an equal isotope concentration) that all carbons of the fatty acid chain in question are converted to  $CO_2$  at equal rates.

As nearly as can be judged from the limited data available in Table IV, all types of labeled acetate are oxidized at about the same rate. The average for acetate is 27 per cent; i.e., 27 per cent of the isotope was recovered in the respiratory  $CO_2$  in  $2\frac{1}{2}$  hours. Likewise that part of the respiratory  $CO_2$  that came from the fatty acid was for all types of acetate the same, the average being 14 per cent of the total  $CO_2$ .

It is apparent that if the carboxyl carbon of acetate were actually converted to  $CO_2$  more rapidly than the methyl carbon these values would have been higher for the carboxyl-labeled acid than for the methyl-labeled acid, and the values for the doubly labeled acetate would have been intermediate. The fact that all types of labeled acetate give the same results in the respiratory  $CO_2$  is in agreement with the predictions of the cycle. Study of the schematic diagram will reveal that, at the end of the second circulation of oxalacetate through the cycle, one-half as much of the carboxyl carbon of the oxalacetate has been derived from the methyl carbon of the acetate as from the carboxyl carbon of the acetate. By further repeated circulation the total contribution of the carboxyl and methyl carbons of acetate to the carboxyl carbons of oxalacetate approaches equality. Since it is the carboxyl carbons of oxalacetate which yield  $CO_2$  on passage through the cycle, the rate of conversion of the methyl carbon of acetate to  $CO_2$  should approach that at which the carboxyl carbon is so converted.

From the foregoing it is concluded that both carbons of acetate are converted to  $CO_2$  at approximately equal rates. This finding, as well as the results of the distribution of the isotope in the glucose, thus seems to be consistent with the suggestion that acetate may be oxidized by the tricarboxylic acid cycle.

It is noteworthy that the conversion of acetate to glycogen via the cycle cannot account for a net increase in glycogen from acetate carbon *per se*, since 2 moles of  $CO_2$  are produced for each mole of acetate that enters the

cycle. This is in agreement with the fact that glycogen is not deposited in the liver of the fasted rat following the feeding of acetate alone. As previously noted, however, the net change in glycogen in an animal may be as much dependent on the effect of the fed compound on the balance of the dynamic equilibrium of the body as it is on whether or not there is a mechanism for *net* transfer of carbons from the compound to the glycogen. The formation of glycogen in the traditional sense (*i.e.*, net glycogen deposition) contrasted with an actual transfer of carbon from a compound to glycogen as measured with isotopes may have no relation. When viewed in terms of actual movement of carbon to glycogen, it is apparent that short chain fatty acids and, in all probability, long chain fatty acids as well can be converted to carbohydrate. Bloch and Rittenberg (22) have shown that a long chain fatty acid (myristic acid) can give rise to acetyl groups in the intact organism. Furthermore it has been reported that acetate enters aspartic acid and glutamic acid, which are considered to be in biological equilibrium with carbohydrates by routes other than  $\text{CO}_2$  fixation (23). These considerations together with present results with acetate indicate that glycogen carbon can be derived from long chain fatty acids.

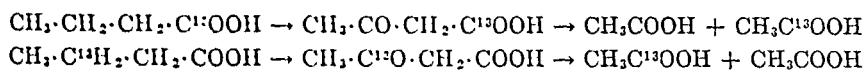
It should be pointed out that the conversion of acetate to succinate by methyl to methyl condensation and a subsequent conversion of the succinate to pyruvate and then to glycogen would meet all the requirements of the present experimental data. Therefore on this basis there is no reason to exclude this mechanism. On the other hand the conversion of acetate to glycogen by a mechanism involving the formation of pyruvate by addition of a 1-carbon compound to the carboxyl of acetate (or a derivative) can be excluded. If this occurred, it would be expected that carboxyl-labeled acetate and probably carboxyl- and  $\beta$ -labeled butyrate would give rise to a  $\alpha$ -labeled pyruvate. This type of pyruvate apparently was not the means of entrance of the acetate, for if it were, one would expect labeling to appear mainly in the 2,5 positions of the glucose. Actually this did not happen to a significant extent. On the other hand the results do not necessarily exclude the addition of a 1-carbon compound to the methyl carbon of acetate.

It is thus seen that, although the results are in agreement with the tri-carboxylic acid cycle as a mechanism of conversion, this is by no means the only mechanism which would explain the results.

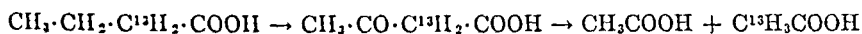
*Pathways from Butyrate to Glycogen*—There is a large body of experimental facts which indicates that fatty acids may be oxidized by  $\beta$  oxidation to acetate or derivatives of acetate (22, 24). If this occurs, it is reasonable to suppose that the resulting acetate would be converted to glycogen. With different types of labeled butyrate it should be possible by comparison with results obtained with the different kinds of labeled



acetate to determine whether or not the  $\beta$  oxidation scheme suitably explains butyrate metabolism in intact animals. Thus carboxyl-labeled butyrate or  $\beta$ -labeled butyrate should be equivalent to carboxyl-labeled acetate and lead to the deposition of glycogen with isotope limited to the 3 and 4 carbon atoms of the glucose unit.

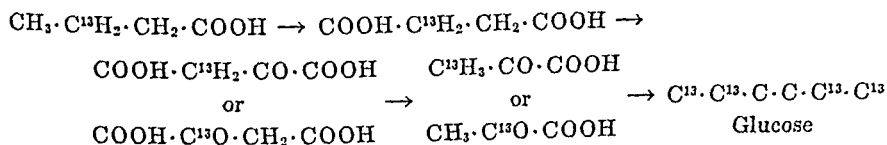


$\alpha$ -Labeled butyrate should be equivalent to methyl-labeled acetate and lead to the deposition of glycogen with isotope preponderantly in carbon atoms 1, 2, 5, and 6.



The results shown in Table II are in complete agreement with these proposals and offer support to the idea that butyrate undergoes  $\beta$  oxidation and that the resulting 2-carbon compound may be converted to glycogen via the tricarboxylic acid cycle through reactions similar to those previously described for acetate. It is realized, of course, that what are observed in these tests are the end-results and that no real information is given on intermediate steps. There may be other mechanisms which would fulfil the requirements of the data, but at present the  $\beta$  oxidation theory appears adequate.

The present results apparently rule out, at least for the conditions of these experiments, the formation of glycogen by  $\omega$  oxidation of butyrate to succinate as suggested by Blixenkrone-Möller (25). Whereas the results with carboxyl- and  $\alpha$ -labeled butyrate could be explained by this mechanism, it cannot explain the results with the  $\beta$ -labeled butyrate. By  $\omega$  oxidation to succinate, isotope from  $\beta$ -labeled butyrate would be located predominantly in positions 1, 2, 5, and 6 of the glycogen, as illustrated by the following equations.



Actually, as already noted, a significant excess of  $\text{C}^{13}$  could be detected only in positions 3 and 4. It should be noted that this distribution probably would not result by  $\omega$  oxidation even if the resulting dicarboxylic acid were not symmetrical. From an unsymmetrical  $\text{C}_4$ -dicarboxylic acid either of the two types of pyruvate as illustrated above would be expected and neither would give a glucose unit labelled in positions 3 and 4. Furthermore the oxidation of butyrate to succinate, followed by cleavage in the

center, to 2 molecules of acetate is not likely, because this mechanism would be expected to give a glucose from  $\beta$ -labeled butyrate like that obtained from methyl-labeled acetate, whereas it did not.

Metabolism of butyrate via acetate would not directly provide for a net increase in carbohydrate precursors, but such an increase if it does occur (as indicated, for example, by the work of Blixenkrone-Möller, wherein glucogenesis was reported in perfused livers following the administration of butyrate) could be an indirect effect of the fatty acid. The presence of butyrate may exert a sparing effect and cause the accumulation of glycogen from available carbohydrate or carbohydrate precursors derived from protein.

If butyrate is metabolized via acetate, one might expect, from the results on the three types of labeled acetate, that all carbon atoms of butyrate would be converted to  $\text{CO}_2$  at approximately equal rates. From the data on respiratory  $\text{CO}_2$  (Table IV) it would appear that the carboxyl and  $\beta$ -carbons of butyrate are burned much more rapidly than is the  $\alpha$ -carbon. Unfortunately, the experiments with the  $\alpha$ -labeled butyrate are not entirely comparable to those in which the carboxyl- and  $\beta$ -labeled acids were fed, since considerably smaller amounts of the  $\alpha$ -labeled butyrate were administered and also this fatty acid was contaminated with appreciable amounts of non-isotopic propionate (see Table I). Both these factors would lower the relative amount of labeled carbon in relation to the total metabolic pool and thus the  $\text{C}^{13}$  in the respiratory  $\text{CO}_2$  would be less concentrated. As a consequence of this, as judged by column (d), Table IV, which gives the dilution in the respiratory  $\text{CO}_2$  of administered isotope by non-isotopic metabolic  $\text{CO}_2$ , the oxidation of the  $\alpha$ -carbon would be indicated to be relatively lower than for the other positions. Actually, however, a proportionally greater oxidation of non-isotopic material may have been the real cause of this difference. The values for the per cent of the administered isotope recovered in the  $\text{CO}_2$  (column (c)) also indicate a slower oxidation of the  $\alpha$ -carbon, but no definite conclusion seems justified on the basis of the two results, for it is seen in column (c) that there is a great deal of variation in the results from one experiment to another. This variation may in part be caused by variation in absorption. Thus at present there is no reliable indication that the rate of oxidation differs for the individual positions of the butyrate molecule and this question must be left open.

Considered as a whole, these studies with acetate and butyrate which have been performed on intact normal animals have yielded results which agree remarkably well with predictions from schemes which have been derived for the most part from studies *in vitro*. The results give some evidence that the tricarboxylic acid cycle is an active mechanism in the normal

animal; also the evidence lends support to the conventional schemes of glycolysis. It should be borne in mind that, although these studies were made on whole, fasted animals, they probably represent in a large part the metabolism of the liver. It is conceivable that studies of longer duration with glycogen from other tissues might yield results which differed from the present results. Conceivably kidney, heart, etc., may follow metabolic pathways with some variations from the metabolism of liver.

#### SUMMARY

The glycogen of rat liver was isolated following feeding, by stomach tube, of glucose plus either acetate or butyrate labeled with  $C^{13}$ . The glycogen was hydrolyzed to glucose, and the latter degraded.

After the administration of  $CH_3 \cdot C^{13}OOH$  or  $CH_3 \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH$  as well as after the administration of  $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot COOH$ , all of the excess isotopic carbon in the glycogen was found in positions 3 and 4 of the glucose unit. Since these are the same positions as for  $CO_2$  fixation, and since considerable amounts of isotopic carbon appeared in the respiratory  $CO_2$ , it was impossible from location of  $C^{13}$  in the glycogen to determine whether there was an additional pathway of conversion of fatty acid carbon to glycogen.

With  $C^{13}H_3 \cdot COOH$  or  $C^{13}H_3 \cdot C^{13}OOH$  or  $CH_3 \cdot CH_2 \cdot C^{13}H_2 \cdot COOH$ , all degradation fractions of the glycogen contained excess  $C^{13}$ . This is considered direct evidence that at least the  $\alpha$ -carbon atoms of acetate and butyrate can enter glycogen by a pathway other than  $CO_2$  fixation.

The distribution of isotope found in liver glycogen is consistent with formation of 3-carbon carbohydrate fragments from acetate and butyrate via the tricarboxylic acid cycle, and synthesis of the 6-carbon glycogen units from two 3-carbon fragments through reversal of the conventional reactions of glycolysis.

The results after the administration of butyrate are consistent with the  $\beta$  oxidation of butyrate to 2 molecules of acetate, but inconsistent with  $\omega$  oxidation of butyrate to succinate.

These observations have been made with intact animals and provide some evidence for the functioning, under *in vivo* conditions, of schemes based largely on *in vitro* studies.

We wish to thank Dr. Walton Shreeve, Miss Carol Carlson, Miss Eleanor Davies, Miss Bernice Theissen, and Miss Margaret Cook for help with a number of the chemical determinations, and Dr. A. O. C. Nier of the Department of Physics, University of Minnesota, for most of the mass spectrometer measurements.

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# INFLUENCE OF TESTOSTERONE PROPIONATE ON THE PLASMA AND LIVER PROTEINS OF HYPOTHYROID RATS\*

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(Received for publication, July 23, 1948)

Clinically a relationship between thyroid activity and blood protein levels has been indicated by a hyperproteinemia in myxedema (1) and is correlated with an increase in globulin levels (2). This relationship has been observed in dogs (3) and has been seen repeatedly in thyroidectomized rats and in rats fed antithyroid drugs (4-6). Since a decrease in weight of the seminal vesicles followed thyroidectomy (7) and thiourea feeding (8), the implication that androgen production is waning in hypothyroidism invited the study of testosterone propionate action in rats fed antithyroid drugs. These data aid in determining whether hypothyroidism or hypogonadism is of primary importance in causing the rise in plasma globulin associated with both conditions. Furthermore, an effect might be anticipated, since androgen administration induced nitrogen retention in a cretin (9).

Liver size and the ratio of liver protein to body weight increased after thiouracil feeding (10). In view of the known protein anabolic action of androgens (11) studies concerning liver protein in the hypothyroid state with and without concomitant androgen are presented.

## EXPERIMENTAL

Male rats of the Long-Evans strain were used when 150 days old and were kept in metabolism cages for measurement of daily food intake. The rats were raised on Purina fox chow (20.1 to 23.9 per cent protein) and it was also fed during the experimental period. Thiourea and thiouracil,<sup>1</sup> were added as 0.5 per cent of the diet, and the rats fed *ad libitum*. Other groups received thiourea or thiouracil and testosterone propionate<sup>1</sup> or were normal rats with and without androgen, but all were pair-fed with the group on the goitrogenic substance alone, as thiourea and thiouracil reduce

\* Supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University, initially, and completed under a contract with the Office of Naval Research, Navy Department.

<sup>1</sup> Thiouracil (deravet) was supplied by Dr. Mark Welsh, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, and testosterone propionate (perandren, Ciba) was supplied by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, New Jersey.

food intake. After a 20 to 27 day experimental period the rats were lightly anesthetized with ether and bled from the heart. Hematocrit, non-protein nitrogen, total plasma protein, albumin, and globulin were determined. Albumin and globulin were separated by the Howe method (12), as modified by Robinson, Price, and Hogden (13). Nitrogen values were corrected for non-protein nitrogen and converted to protein by use of the factor 6.25. Liver nitrogen was determined after the organ had been dried to constant weight at 95° and ground to uniform consistency.

### Results

Thiourea feeding reduced food intake to an average of 273 gm. per rat for 20 days, as compared to the 335 gm. consumed by rats eating *ad libitum*. The reduced food intake resulted in a loss in body weight which, however, was greater in the thiourea-fed rats than in pair-fed controls. Testosterone propionate administered subcutaneously at a level of 0.1 mg. daily did not prevent weight loss, although this androgen caused retention of urinary nitrogen in men on a restricted caloric intake (14).

Thiourea-fed rats exhibited a decrease in hematocrit and an increase in non-protein nitrogen, but an increase was not observed in normal rats on reduced food intake. Of the deviations from normal in rats fed thiourea, only non-protein nitrogen remained within the normal range in rats receiving both thiourea and androgen. Total plasma protein was increased by thiourea, owing to an increase in concentration of plasma globulin, while plasma albumin levels were unaltered (Table I). The androgen had a tendency to reduce the elevated plasma protein levels, but the differences were not significant. In the normal rat on restricted food intake, the plasma globulin level had a tendency to be higher than in rats fed *ad libitum*, being 2.72 gm. per cent compared with 2.51 gm. per cent. Androgen administration reduced the plasma globulin to a normal concentration, but in all cases the data are of border line significance.

Although the daily dosage of 0.1 mg. of testosterone propionate was adequate in maintaining weight of the seminal vesicles in castrated rats, this dosage was essentially ineffective in maintaining normal plasma protein levels in hypothyroid rats. It seemed advisable to repeat the entire experiment with 0.5 mg. of androgen daily, but the results generally duplicated the data obtained with the lower dosage.

The possibility that a toxic action of thiourea may have masked the action of testosterone on the concentration of plasma protein prompted the use of thiouracil. Thiouracil fed at 0.5 per cent reduced food intake to 300 gm. per rat over 20 days, causing a modest loss in body weight, which was duplicated by pair-fed controls. 0.5 mg. of testosterone propionate was used alone and in combination with thiouracil. Loss in body

weight was not prevented by testosterone propionate, but the increase in non-protein nitrogen and the decrease in hematocrit induced by thiouracil could be largely prevented by androgen administration. Thiouracil in-

TABLE I

*Plasma Protein Concentrations in Rats Treated with Thiourea (0.5 Per Cent) and Testosterone Propionate (0.1 Mg.)*

No. of rats	Treatment	Body weight*	Hematocrit	Non-protein nitrogen	Total protein	Albumin	Globulin
		gm.	per cent	mg. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
12	Thiourea	294-256	40.5 ±1.0†	68 ±3.4	6.66 ±0.10	3.49 ±0.12	3.17 ±0.09
12	" + testosterone propionate	294-259	41.3 ±1.1	60 ±3.2	6.41 ±0.11	3.38 ±0.15	3.02 ±0.09
12	Normal, pair-fed	288-271	45.4 ±0.8	58 ±3.2	6.10 ±0.07	3.37 ±0.06	2.72 ±0.09
6	" + testosterone propionate	303-280	46.3 ±1.2	57 ±4.0	5.93 ±0.07	3.36 ±0.14	2.57 ±0.14

\* Initial and final.

†  $\epsilon = \sqrt{\Sigma d^2 / (n(n-1))}$ .

TABLE II

*Plasma Protein Concentrations in Rats Treated with Thiouracil (0.5 per cent) and Testosterone Propionate (0.5 Mg.)*

Each group consisted of twelve rats.

Treatment	Body weight*	Hematocrit	Non-protein nitrogen	Total protein	Albumin	Globulin
	gm.	per cent	mg. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Thiouracil	336-326	44.4 ±0.5	66 ±1.7	6.81 ±0.09	3.26 ±0.08	3.55 ±0.17
" + testosterone propionate	331-317	46.4 ±0.6	58 ±2.5	6.95 ±0.10	3.36 ±0.07	3.59 ±0.13
Normal, pair-fed	336-315	48.7 ±0.6	56 ±1.1	6.04 ±0.11	3.19 ±0.08	2.85 ±0.14
" + testosterone propionate	324-312	49.7 ±0.7	54 ±2.7	6.08 ±0.08	3.23 ±0.10	2.85 ±0.09

\* Initial and final.

creased plasma globulin concentrations in the absence of a change in plasma albumin levels (Table II), and the administration of androgen was without effect. The increase in plasma globulin in rats due to thiouracil is associated with an increase in  $\alpha$ -globulin (6), and  $\alpha$ -naphthylthiourea

incites a similar reaction in dogs (15). The antithyroid drugs have varied toxicities, and thus their action might be to stimulate the adrenal, the secretions of which increase serum globulin (16). The action of antithyroid drugs on plasma protein levels would seem to be independent of adrenal excitation, however, since these compounds reduce adrenal activity (17, 18). Furthermore, normal globulin levels can be maintained in hypophysectomized rats with thyroxine (4), and adrenocorticotrophic hormone does not release globulin in these animals (19).

Table III reveals that thiouracil will increase liver weight significantly without changing the percentage of water or protein. Consequently the total liver protein in the body and the liver protein in gm. per 100 gm. of body weight were significantly above normal. Testosterone propionate

TABLE III

*Influence of Thiouracil (0.5 Per Cent) and Testosterone Propionate (0.5 Mg.) on Liver of Adult Rats*

Each group consisted of seven rats.

Treatment	Liver weight		Water per cent	Total protein gm.	Liver protein	
	gm.	gm. per 100 gm. body weight			per cent dry weight	gm. per 100 gm. body weight
Thiouracil	15.2	4.4	71.0	2.93	66.9	0.887 (0.801-0.999)
	±0.9	±0.3	±0.3	±0.13	±1.2	
“ + testos- terone propionate	13.2	4.2	70.3	2.63	67.2	0.835 (0.714-0.910)
	±0.7	±0.3	±0.2	±0.11	±1.2	
Normal, pair-fed	10.9	3.3	70.1	2.27	69.9	0.719 (0.652-0.788)
	±0.6	±0.2	±0.6	±0.10	±1.7	
“ + testos- terone propionate	10.9	3.5	69.9	2.27	70.1	0.717 (0.626-0.856)
	±0.9	±0.3	±0.3	±0.13	±1.5	

was essentially without effect on the liver water or protein in thiouracil-fed or normal pair-fed rats. The increase in liver weight induced by thiouracil was less pronounced when androgen was administered concomitantly. The increase in the ratio of liver protein to body weight is obtained only after thiouracil feedings (10), thyroidectomy favoring liver atrophy (20). The thiouracil action is surprising in the wake of reduced food intake, since the liver can be depleted of protein quickly (21). The increase in liver weight might also be aided by an increase in fat (22) and glycogen (23), but contradictory data have also been reported regarding these components in the liver (23, 18).

## SUMMARY

Hypothyroidism induced by feeding thiourea or thiouracil resulted in an increased concentration of total plasma protein, plasma globulin, and



non-protein nitrogen. Plasma albumin concentration was not changed, but the hematocrit decreased. An increase in liver size, while water and protein remained at normal percentages, resulted in an increased liver protein in the body after thiouracil feeding. Testosterone propionate in doses of 0.1 and 0.5 mg. daily did not alter the plasma or liver proteins of hypothyroid rats, except to reduce the non-protein nitrogen. The slight rise in plasma globulin sometimes associated with restricted food intake was prevented by testosterone propionate administration, and the increase in liver weight induced by thiouracil was less pronounced when androgen was administered concomitantly.

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# STUDIES ON THE STRUCTURE OF LYCOMARASMIN

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(Received for publication, July 3, 1948)

Plattner and Clauson-Kaas (1) have isolated a substance called lycomarasmin from the culture filtrates of the phytopathogenic fungus *Fusarium lycopersici*. When this organism infects tomato plants, it causes the leaves to wilt and curl, and this is believed to be due to the formation of a toxin which can be demonstrated in the intercellular spaces of the host (2). A similar toxin is formed by the fungus growing in synthetic medium, because filtrates of such cultures cause excised tomato leaves to wilt and curl just as they do on infected plants. The lycomarasmin which was isolated from these filtrates had the same powers against tomato leaves and represented a considerable portion of the toxic activity of the culture.

Plattner and Clauson-Kaas (3) found that lycomarasmin was a small molecule of empirical formula  $C_9H_{15}O_7N_3$ , and that it yielded on hydrolysis two amino acids, glycine and aspartic acid. Since these constituents would leave only 3 carbon atoms and 1 nitrogen atom unaccounted for, and since mild hydrolysis led to the formation of 1 molecule of ammonia, a labile grouping was indicated in the molecule, and indeed, the failure of the toxin to give a blue color with ninhydrin, or  $N_2$  in the Van Slyke reaction with  $HNO_2$ , suggested that the unidentified 3 carbon and 1 nitrogen did not belong to a conventional amino acid such as alanine. The instability of lycomarasmin was well illustrated by the finding that heating it in water, or merely allowing the free acid to stand for long periods in aqueous solution, led to the formation of a biologically inactive compound,  $C_9H_{12}O_7N_2$ , which differed from the toxin by the elements of  $NH_3$ . This latter compound was designated Substance I. Largely on the basis of these findings, a structure was proposed for lycomarasmin (Fig. 1).

Since strepogenin had been shown to be quite probably a derivative of glutamic acid (4), the hypothesis occurred to us that lycomarasmin and strepogenin might be structural analogues in which the glutamic acid of the growth factor strepogenin was replaced by aspartic acid in the inhibitory or toxic lycomarasmin. The occurrence of glycine in both substances (3, 5) was interesting in this connection. Therefore, an investigation of the structure of lycomarasmin was begun in this laboratory in the hope of throwing light on the constitution of strepogenin. Lycomarasmin had been obtained in pure form, whereas strepogenin had not.

The results of these studies do not seem to be compatible with the struc-

ture proposed by Plattner and Clauson-Kaas (3), which was admittedly tentative. In the first place, lycomarasmin can be shown to be a derivative of asparagine. If the toxin is treated with a solution of K<sub>2</sub>OBr, a Hofmann degradation apparently occurs, and aspartic acid can no longer be found in

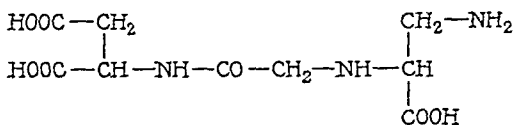
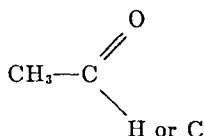


Fig. 1. Structure of lycomarasmin according to Plattner and Clauson-Kaas

the hydrolytic products. Substance I of Plattner and Clauson-Kaas, on the other hand, maintains its aspartic acid after treatment with hypobromite. The location of the 3rd N atom thus is indicated. In the second place, lycomarasmin yields iodoform, whereas Substance I does not. Therefore, the grouping



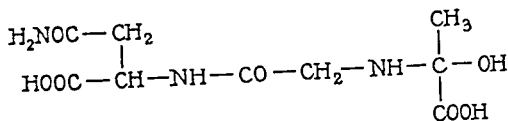
must be present in (or derivable from) the former but absent from the latter. As Plattner and Clauson-Kaas (1) have indicated, no free aliphatic NH<sub>2</sub> group seems to be present, so that when either lycomarasmin or Substance I is treated with HNO<sub>2</sub>, neither glycine nor aspartic acid is destroyed. When lycomarasmin is tosylated with *p*-toluenesulfonyl chloride in warm, alkaline solution, a simultaneous cleavage takes place, and an ethyl acetate-soluble tosyl derivative is formed. On careful hydrolysis this derivative yields tosylglycine and aspartic acid, from which it may be concluded that the tosyl derivative is probably tosylglycylaspartic acid. The unstable 3 C moiety of the toxin is probably lost during the acylation. Substance I apparently contains a double bond, because it adds bromine in the cold to give a halogenated derivative.

In view of these facts, the structures of Fig. 2 are proposed to represent lycomarasmin and Substance I. The toxin thus would contain a derivative of the unstable amino acid,  $\alpha$ -hydroxyalanine. This is stabilized in such a way as not to lose ammonia by the replacement of 1 hydrogen atom of its amino group by the nitrogen-free part of a glycine residue. This new and unstable amino acid has been suspected to occur in another product of fungi, namely ergotamine, as demonstrated by Jacobs and Craig (6).

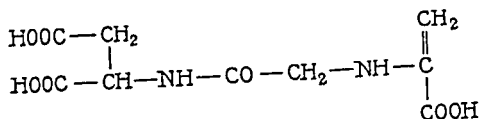
A second motive which stimulated the work was the realization that lycomarasmin was a small molecule, composed of amino acid residues,

with marked biological activity. It might thus serve well as a model for the study of some aspects of the structure and biological activities of proteins. Being a small molecule, it could be subjected to searching proof of structure and to chemical rearrangement of the component parts by totally synthetic means much more readily than is true for the proteins.

The easy conversion of lycomarasmin into Substance I merely by heating or standing is an interesting analogy to heat-inactivation of more complex peptides or proteins. Thus the active toxin is converted to an inactive product in the reaction. If the formulation in Fig. 2 is correct, the chemical change in this case would involve the loss of water between the unstable tertiary alcoholic group and the H of the adjoining methyl group. The



Lycomarasmin



Substance I

FIG. 2. Proposed structures for lycomarasmin and for Substance I of Plattner and Clauson-Kaas.

ammonia which is liberated during the reaction would arise by concomitant hydrolysis of the amide group of the asparagine residue.

An attempt was made to synthesize lycomarasmin as represented in Fig. 2 by the condensation of ethyl- $\alpha$ -acetoxy- $\alpha$ -bromopropionate with the methyl ester of glycylasparagine, followed by selective saponification of the ester groups. A substance was isolated from the reaction mixture which had approximately the theoretical amount of nitrogen for lycomarasmin. Furthermore, its biological activity in causing wilting and curling of tomato leaves was equal to that of lycomarasmin. However, too much weight cannot be given this latter finding, because, as will be shown in a subsequent paper, several substances closely related to lycomarasmin possess biological activity. The difficulty in establishing beyond question the identity of the synthetic product with lycomarasmin was that the proposed structure contained 2 asymmetric carbon atoms, and although optically active asparagine was used in the synthesis, the bromoacetoxypropionate was racemic. The

rather unstable nature of this compound prevented adequate purification or resolution. Therefore, the synthetic product must have been a mixture of diastereomers, and this probably explains the observed fact that the synthetic substance was more soluble in water than was the natural product.

#### EXPERIMENTAL

*Source of Material*—Crystalline lycomarasmin and Substance I were isolated according to the directions of Plattner and Clauson-Kaas (1) from 3 month-old culture filtrates of *Fusarium lycopersici*. A transplant of this organism was very kindly supplied by Dr. G. W. Irving, Jr., of the United States Department of Agriculture. The substances agreed well in all physical properties with those described by Plattner and Clauson-Kaas.

*Amino Acid Composition of Lycomarasmin and Substance I*—In order to ascertain whether glycine and aspartic acid were the only amino acids liberated on acid hydrolysis of lycomarasmin and Substance I, 5 mg. samples were digested in sealed tubes with 22 per cent HCl at 115° for 20 hours. The hydrolysates were concentrated to dryness under reduced pressure, dissolved in water, again concentrated, and finally dissolved, neutralized, and adjusted to 5 cc. These preparations were then partitioned on paper strips according to the directions of Consden, Gordon, and Martin (7). With either butanol or with phenol as the organic phase, only two colored zones, corresponding to the positions of glycine and aspartic acid, were found. Quantitative estimation of the amounts of these two amino acids by microbiological methods was not satisfactory because of the great difficulty experienced in obtaining complete hydrolysis. As the rigor of the treatment was increased, the yield of amino acids went up, so that with the conditions outlined in the paragraph above, almost twice as much of both amino acids were found as with hydrolysates prepared by refluxing with 20 per cent HCl overnight. Even with the more vigorous hydrolysis, Substance I yielded values of 30 per cent for aspartic acid and 17 per cent for glycine. Theory, 51 and 29. The aspartic acid was estimated by the method of Hac and Snell (8). At the time these experiments were being performed, no microbiological method for the determination of glycine had been published, but a satisfactory one was devised by use of the same organism and basal medium as were employed for the aspartic acid determination. Glycine was omitted and aspartic acid was added to the stock solutions. In addition, 0.1 mg. of an acid hydrolysate of casein was added per tube. The use of the organism was suggested by Dr. M. S. Dunn, who has since published a similar method for the microbiological assay of glycine (9). Despite the difficulty of obtaining complete liberation of amino acids from the peptides lycomarasmin and Substance I, the molar ratio of aspartic acid to glycine in the hydrolysates was 1:1.

*Hofmann Degradation of Lycomarasmin and of Substance I to Demonstrate Presence of Substituted Asparagine in Former*—2.5 mg. samples of lycomarasmin and of Substance I were dissolved in 2.5 cc. of water at pH 7, and the solutions were cooled to 0° and mixed with a cold solution of 100 mg. of bromine in 2 cc. of  $N$  KOH. The reaction was allowed to proceed for half an hour at 0°, and then for an hour at 40°. A slight excess of HCl was added, and the solutions were evaporated to dryness under reduced pressure. The residues were taken up in 22 per cent HCl, hydrolyzed, and assayed for aspartic acid. At the same time, controls were run in which the KOH but not the bromine was used.

Under these conditions, no aspartic acid was found in the reaction product from lycomarasmin, while that from Substance I retained about 75 per cent of this amino acid. When asparagine and aspartic acid were treated with KOBr in the same fashion, all of the former and about a third of the latter were destroyed.

*Iodoform from Lycomarasmin*—25 mg. of lycomarasmin were dissolved in 2 cc. of 2  $N$  NaOH, and the solution was treated with iodine dissolved in 15 per cent aqueous KI until a yellow color persisted. The mixture was then warmed to 60° for a few minutes. During the warming, a yellow precipitate formed, and the odor of iodoform was plainly discernible. The precipitate was found to consist of yellow, hexagonal plates which melted at 119°.

When 100 mg. of Substance I were tested in the same fashion, no evidence of iodoform was obtained.

*Failure to Destroy Glycine or Aspartic Acid by Treatment of Lycomarasmin with  $HNO_2$* —20 mg. of lycomarasmin dissolved in 1 cc. of water plus 3 cc. of glacial acetic acid were treated with 35 mg. of  $NaNO_2$  dissolved in 2 cc. of water. After a reaction time of 0.5 hours had elapsed, excess reagents were destroyed by evaporation under reduced pressure and by addition of 40 mg. of alanine. Hydrolysis and estimation of glycine and aspartic acid were carried out as indicated earlier, and 4.8 mg. of the former and 7.2 mg. of the latter were found. A control in which the  $NaNO_2$  was omitted was found to yield the same values. Therefore, the amino groups of glycine and of aspartic acid were not free in lycomarasmin.

Control experiments showed that under these conditions, both free glycine and free aspartic acid were so altered by  $HNO_2$  as not to be determinable in the assays. Furthermore, it was observed that HCl could not be used successfully in place of acetic acid in the complete deamination of these substances.

*Isolation of Tosyl Glycine by Tosylation and Subsequent Partial Hydrolysis of Lycomarasmin*—200 mg. of lycomarasmin were dissolved in water at pH 7, and 12 cc. of 1  $N$  NaOH were added and the mixture heated to 70°. 1 gm. of *p*-toluenesulfonyl chloride was added, and the suspension was

shaken until the acid chloride had disappeared (about 5 minutes). The cooled, acidified reaction mixture was extracted six times with ethyl acetate, and the extract was freed of solvents under reduced pressure. The material so obtained was a water-soluble substance which could not be crystallized. It was, therefore, dissolved in 13 cc. of 0.5 N HCl and 13 cc. of formic acid, and the solution was refluxed for 48 hours. The solvents were removed under reduced pressure, and the residue taken up in 5 cc. of water. A crystalline precipitate was removed and the aqueous phase was extracted four times with ethyl acetate. The extracted material was combined with the crystals and the whole recrystallized from water to yield 73 mg. melting at 142°, the same as did tosylglycine.

$C_9H_{11}O_4NS$ . Calculated, C 47.2, H 4.8, N 6.1; found, C 47.2, H 4.9, N 6.1

The aqueous phase from the hydrolysate was assayed for aspartic acid and found to contain 51 mg. of it. It seemed probable that a 3 carbon moiety was cleaved under the conditions of the acylation, and that the oily product subsequently extracted by ethyl acetate was largely tosylglycyl-aspartic acid.

*Synthesis of Substance with Biological Activity and Several Other Properties of Lycomarasmin.* (a) *Ethyl- $\alpha$ -acetoxy- $\alpha$ -bromopropionate*—6.6 gm. of acetyl-lactic acid and 2 gm. of red phosphorus were treated dropwise, with stirring and cooling, with 16 gm. of bromine. After the rather vigorous reaction, with evolution of HBr, had subsided, the mixture was allowed to stand overnight. Excess bromine and HBr were removed under reduced pressure, and 100 cc. of cold water were added. The free acid was extracted with ether, and the solvent was removed from the extract under reduced pressure. The yellow, oily product (7.7 gm.) could not be purified further because of its tendency to decompose. It showed a neutral equivalent of 191, whereas theory for  $\alpha$ -acetoxy- $\alpha$ -bromopropionic acid was 211. In order to determine whether any of the desired substance was present, 240 mg. were dissolved in 5 cc. of 1 N NaOH and held at 90° for 1 hour. Acidification, extraction of the acids, and preparation of the *p*-nitrophenyl-hydrazone gave the characteristic derivative of pyruvic acid which melted at 210–213°. Hydrolysis of the acetyl group of  $\alpha$ -acetoxy- $\alpha$ -bromopropionic acid should lead to an unstable intermediate which, by loss of HBr, should yield pyruvic acid.

The ethyl ester was prepared by allowing the acid to stand in a large excess of absolute alcohol for 3 months at 4°, but a better procedure was to add 20 cc. of absolute alcohol to the reaction mixture directly after the bromination before any moisture was admitted. The crude ester was then collected in ether, washed with water, dried, and freed of solvents and



volatile impurities *in vacuo*. The product was a neutral yellow oil which slowly liberated acid on exposure to water.

(b) *Condensation with Methyl Ester of Glycylasparagine*—4 gm. of the above bromo ester, dissolved in 10 cc. of absolute methanol, were added to a methanol solution of the methyl ester of glycylasparagine which had been prepared from 3.8 gm. of free glycylasparagine.<sup>1</sup> The mixture was brought to pH 8 with sodium methoxide, concentrated to 20 cc., and allowed to stand overnight at room temperature. 10 cc. of aqueous 4 N NaOH and 30 cc. of methanol were added, and after 1 hour excess alkali was neutralized with acetic acid. A solution of 10 gm. of barium acetate in 100 cc. of water was added, and the concentration of methanol was adjusted to 70 per cent. The barium salts were allowed to separate overnight in the cold, and they were then collected, washed, and dissolved in 150 cc. of water and reprecipitated with 450 cc. of methanol. They were finally dissolved in water, freed of barium ion exactly with sulfuric acid, and the solution so obtained was concentrated under reduced pressure below 40° to a small volume. Addition of alcohol produced a white precipitate, which was allowed to form for several days in the cold and was then collected and washed. 410 mg. of substance were obtained which were purified by solution in water and reprecipitation with alcohol.

$C_9H_{15}O_7N_2$ . Calculated, N 15.2; found, N 15.5

One-third of the nitrogen was amide nitrogen which was liberated as ammonia during hydrolysis with 1 N HCl. Found, 5.25; calculated, 5.07. The substance gave many of the reactions shown by lycomarasmin, such as the iodoform test and the formation of a white, highly insoluble precipitate when the dry material was suspended in 1 N NaOH. It was an acid which dissolved readily in NaOH and did not give this white precipitate if a large excess of alkali was avoided. The material differed from natural lycomarasmin in that it was more soluble in water. Distinctive derivatives of lycomarasmin are lacking, so that precise comparison was not possible. The synthetic substance had the same biological potency as natural lycomarasmin, whereas glycylasparagine and glycylaspartic acid gave no effect at 25 times the concentration.

<sup>1</sup> This ester was prepared by suspending 3.8 gm. of glycylasparagine in 50 cc. of anhydrous methanol which was then saturated with dry HCl. 100 cc. of methanol were added and the mixture was held at 4° for 2 days. The solvent was then removed under reduced pressure at 4° and excess HCl was eliminated by several additions of methanol, followed by distillation under reduced pressure. The product was then dried in a vacuum desiccator over KOH. The free ester was obtained by suspension of the hydrochloride in methanol and addition of the theoretical amount of sodium methoxide dissolved in methanol. Sodium chloride was removed by filtration.

*Biological Assay with Tomato Leaves*—The synthetic substance was assayed for ability to cause wilting and curling of excised tomato leaves, and it was found that 0.2 mg. per 7 cc. of test medium was the minimal effective amount. Lycomarasmin showed the same value in a series of parallel assays. The conditions of the test were those described by Clauson-Kaas *et al.* (1, 2). In order to improve the accuracy of the test, it was found important to select leaves of uniform size and age and to conduct the experiments in a room provided with controlled illumination and with constant temperature and humidity (50 per cent). Graded 2-fold dilutions of the material under test were supplied to the leaves, and the results were read after 40 hours. Severity of wilting and curling was then judged, and the minimal effective dose which would just cause detectable alteration of the leaves was taken as the end-point. Activity was not estimated solely by the minimal effective dose, but rather by the responses to larger amounts as well. The synthetic and natural products seemed to be equal in potency.

#### SUMMARY

Lycomarasmin, a tomato leaf-wilting toxin isolated by Plattner and Clauson-Kaas from culture filtrates of the phytopathogenic fungus *Fusarium lycopersici*, has been studied in an effort to arrive at a satisfactory chemical structure for it. This small, peptide-like toxin, of empirical formula  $C_9H_{15}O_7N_3$ , which yielded glycine and aspartic acid on hydrolysis, was shown to be a derivative of asparagine. Thus the 3rd N atom, which had previously not been assigned position, was located. Because of the reactions which lycomarasmin underwent, a structural formula for it was proposed which contained the new amino acid,  $\alpha$ -hydroxyalanine. This was attached by a common nitrogen atom (*i.e.*, the amino group) to the amino group of glycylasparagine. A synthetic product was isolated from the reaction of ethyl- $\alpha$ -acetoxy- $\alpha$ -bromopropionate and the methyl ester of glycylasparagine, which had properties in common with lycomarasmin, including quantitatively the same biological activity. Some reasons were advanced for regarding lycomarasmin as a much simplified model of proteins.

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# SYNTHESIS AND DETERMINATION OF LYCOMARASMIN ACTIVITY OF SOME DERIVATIVES OF ASPARTIC ACID

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(Received for publication, August 6, 1948)

Since the phytopathogenic toxin of *Fusarium lycopersici*, first isolated by Plattner and Clauson-Kaas (1), has been shown by Woolley (2) probably to have the structure of N-( $\alpha$ -( $\alpha$ -hydroxypropionic acid))-glycylasparagine, the synthesis and determination of the activity of a number of closely related compounds became of interest. Not only would such a study add to our knowledge of the relationship of structure to biological activity, but more especially it would allow investigation on a much simplified scale of some problems concerned with the specific activities of proteins. Since lycomarasmin is a very small peptide with marked activity, the determination of how its biological properties are altered by rearrangement of the constituent amino acids, or by substitution of new groups, might serve as a useful starting point for understanding of similar phenomena connected with the specificity of protein action. Therefore, a number of new compounds related to lycomarasmin, as well as some previously known ones, have been prepared and tested for their ability to cause wilting and curling of excised tomato leaves. Their potency in this regard has been compared with that of the natural toxin.

## EXPERIMENTAL

*Sources of Known Compounds*—Lycomarasmin was isolated from culture filtrates of *Fusarium lycopersici* according to the directions of Plattner and Clauson-Kaas (1). Aspartic acid, asparagine, serine, glycine, and glutathione were commercial samples. Pyruvylglycine was made according to the directions of Bergmann and Grafe (3), and glycylaspartic acid and glycylasparagine by modifications of published methods (4). These modifications were the use of excess of aspartic acid or of asparagine in the reactions with chloroacetyl chloride, and the removal of traces of chloride ions from the glycyl compounds with silver acetate. By using an excess of the amino acid during the acylation, contamination of the product with chloroacetic acid was minimized. The chloroacetylaspartic acid was extracted into ethyl acetate from aqueous solution, and then readily crystallized. The chloroacetylasparagine was not extractable with this solvent, so that the reaction mixture was first purified by removal of ethyl acetate-

\* With the technical assistance of R. Brown and A. Holloway.

soluble matter and then by evaporation of the aqueous phase and solution of the desired product in acetone. It was finally purified by crystallization according to published methods. Syntheses of serylglycylaspartic acid, serylglycylglutamic acid, glycylserylglutamic acid, and alanylglycylglutamic acid have been described previously (5, 6). Serylglycylaspartic acid was a diastereomeric mixture prepared from DL-serine and L-aspartic acid. In addition, a sample was made from DL-serine and DL-aspartic acid in order to compare the relative potencies of the two pairs of isomers.

*Glycylserylaspatic Acid*.—Tosylglycyl-DL-serine azide was coupled with diethyl-L-aspartate, and the free peptide was formed in the manner described for the isomeric serylglycylaspartic acid (6).

$C_9H_{15}O_7N_3$ . Calculated, N 15.2; found, N 15.0

The product was a mixture of diastereomers, since DL-serine had been used.

*Glycylaspartylserine*. (a) *Chloroacetylasparylserine*.—Chloroacetyl-L-aspartic acid was converted to the anhydride by dissolving 16.8 gm. of it in 100 cc. of acetic anhydride and refluxing the solution for 2 minutes. It was then concentrated under reduced pressure to a sirup, which was caused to crystallize by addition of 10 cc. of dry chloroform. Precipitation was completed by further addition of 50 cc. of absolute ether. The anhydride so obtained was converted to the monoethyl ester by dissolving it in 50 cc. of hot absolute ethanol, cooling, and allowing the solution to stand for 24 hours at room temperature. The monoester was purified by removing the alcohol under reduced pressure, dissolving the oily residue in absolute ether, and extracting the latter solution with 150 cc. of ice-cold 5 per cent aqueous  $NaHCO_3$  solution. The acidic half-ester in the aqueous phase was quickly liberated with 1 equivalent of HCl and extracted into fresh ether. This ether extract was then dried rapidly with  $MgSO_4$ , filtered, and freed of solvent under reduced pressure. It was thus secured as 8.9 gm. of a colorless oil. By analogy with the behavior of the corresponding tosyl derivative (7), it was probably mainly the  $\alpha$ , rather than the  $\beta$  ester.

The chloroacetylmonoethylaspartate obtained as described above was dissolved in 100 cc. of absolute ether and treated with 9.0 gm. of  $PCl_5$  for about 30 minutes. The clear solution was then decanted, and the ether was removed under reduced pressure at low temperature. The  $POCl_3$  was extracted from the residue with cold ( $-10^\circ$ ) dry petroleum ether, whereupon the acid chloride crystallized as large rosettes. Without further purification, it was suspended in 100 cc. of absolute ether, and to the solution were added 5.2 gm. of DL-serine dissolved in 60 cc. of ice-cold 1 N NaOH. The mixture was shaken vigorously and maintained faintly pink to phenolphthalein by frequent additions of cold 4 N NaOH. The reaction was allowed to proceed for an hour in the cold, and then the mixture was acidi-

fied to pH 2.5 with HCl, and the ether layer was discarded. The aqueous phase was extracted four times with ethyl acetate, and the extracts were discarded. The desired product was then obtained by extracting the aqueous layer five times with butanol, and the combined extracts were dried with silica gel, filtered, and washed twice with 10 cc. of water. The peptide was then extracted into aqueous solution with enough NaOH to give a final pH of 7. Mild alkaline hydrolysis of an aliquot of this solution, followed by back titration, showed that some ethyl ester of the required peptide probably still remained mixed with the free acid. Therefore 10 cc. of 1 N NaOH were added, and, after 1 hour, this was acidified with 1 equivalent of HCl. The reaction mixture was then concentrated under reduced pressure at a temperature not above 30° to a small volume and treated with 10 volumes of acetone. The acetone-soluble portion yielded 3.5 gm. of an oil, which was caused to crystallize by cautious addition of ether.

$C_7H_{13}O_7N_2Cl \cdot H_2O$ . Calculated, N 8.9; found, N 8.9

(b) *Glycylaspartylserine*—3.5 gm. of the compound just described were dissolved in 250 cc. of concentrated aqueous ammonium hydroxide, and after the solution had stood 3 days at room temperature, it was concentrated under reduced pressure to about 10 cc. and treated with 200 cc. of absolute ethanol. The solid which was thus produced was centrifuged and washed with alcohol. In order to free it of chloride ions, it was dissolved in water, treated with an excess of silver acetate, and the filtrate from the mixture was freed of silver ions with  $H_2S$ . When the concentrated aqueous solution of the product was treated with alcohol, a flocculent precipitate (2.1 gm.) was formed, which was too high in N to be the desired tripeptide. Therefore, it was dissolved in water, brought to pH 8 with barium hydroxide, and the barium salt was crystallized by addition of 2 volumes of methanol. When barium ions were exactly removed from this insoluble salt with sulfuric acid, the free peptide was precipitated as a white powder when alcohol was added to a concentrated aqueous solution of it.

$C_9H_{15}O_7N_3$ . Calculated. N 15.2, aspartic acid 48  
Found. " 15.1, " " 47

Aspartic acid was estimated microbiologically according to the directions of Hac and Snell (8), following acid hydrolysis of the peptide.

*Acetyllactylglycylasparagine*—9.5 gm. of glycyl-L-asparagine, prepared as indicated above, were acylated in cold, alkaline aqueous solution with an ether solution of 15 gm. of acetyllactyl chloride. The conditions of the condensation were like those described in the preceding section for the acylation of serine.<sup>1</sup> After a reaction time of 1 hour, the mixture was acidified to pH 2.5 with HCl, and the aqueous phase was extracted three times

with ether and three times with ethyl acetate. The extracts were discarded, and the aqueous phase was concentrated under reduced pressure, and below  $40^{\circ}$ , to a sirup. This was extracted with 500 cc. of acetone, and the soluble portion was concentrated to a thin sirup which was extracted with a few cc. of acetone. This operation caused the separation of a gummy precipitate which was collected and dissolved in 40 cc. of methanol. When chloroform was added to this solution, crystallization began. A yield of 13.8 gm. of hygroscopic, large crystals was obtained.

$C_{11}H_{17}O_7N_3$ . Calculated, N 13.9; found, N 13.9

This compound was prepared as a starting material for the synthesis of pyruvylglycylasparagine, for it was hoped to remove the acetyl group by mild alkaline hydrolysis and then to oxidize the secondary alcohol to a ketone. Although the cleavage of the acetyl group seemed to proceed readily at room temperature in a slight excess of aqueous NaOH, the product obtained was amorphous and very hygroscopic. For these reasons a satisfactory preparation could not be made. Attempted oxidation of the hydrolyzed product with Fenton's reagent for the formation of the pyruvyl compound gave no desired substance which could be purified satisfactorily.

*$\alpha,\alpha$ -Diacetaminopropionylglycylasparagine*—8.5 gm. of the azlactone of  *$\alpha,\alpha$ -diacetaminopropionic acid (3)* were suspended in 150 cc. of cold alcohol and immediately mixed with an ice-cold solution of 9.5 gm. of glycyl-L-asparagine in 52 cc. of 1 N NaOH. The resulting solution was kept at room temperature for 30 minutes and then acidified with 1 N  $H_2SO_4$  equivalent to the NaOH used, and concentrated under reduced pressure to dryness below  $40^{\circ}$ . The residue was dried by adding 100 cc. of absolute alcohol and again concentrating it under reduced pressure. It was then extracted with 400 cc. of boiling ethanol, and the mixture was filtered hot. A slight precipitate which formed when the filtrate was cooled was discarded, and the alcoholic solution was concentrated under reduced pressure to about 30 cc. and treated with 100 cc. of ethyl acetate. 10 gm. of the product crystallized, and this was recrystallized by adding ethyl acetate to an alcoholic solution. The purified material sintered at  $140^{\circ}$  and melted at  $185$ – $187^{\circ}$ .

$C_{13}H_{21}O_7N_5$ . Calculated. C 43.5, H 5.9, N 19.5  
Found. " 43.5, " 6.2, " 19.0

The neutral equivalent was 370, while the calculated value was 359.

*$\alpha$ -Hydroxy- $\alpha$ -acetaminopropionylglycylaspartic Acid*—1 gm. of diacetaminopropionylglycylasparagine was dissolved in 100 cc. of 0.2 N HCl, and the solution was refluxed for an hour and cooled. It was then adjusted to pH 8 with barium hydroxide, treated with 3 volumes of methanol, and kept in the cold overnight. The precipitate which had formed was collected and

washed well, and then dissolved in water for the exact removal of barium ions with sulfuric acid. The resulting aqueous solution was concentrated under reduced pressure below 40° to a sirup, which was extracted with alcohol in order to free it of pyruvylglycylaspartic acid (75 mg.). The alcohol-soluble portion was freed of solvent, dissolved in water, and adjusted to pH 8 with barium hydroxide. The addition of 2 volumes of methanol and storage in the cold produced a network of crystals, and these were collected, washed, and dried; yield, 264 mg.

$C_{11}H_{13}O_3N_3Ba \cdot H_2O$ . Calculated. C 28.0, H 3.6, N 8.9, Ba 29.2  
Found. " 25.2, " 3.4, " 8.4, " 30.7

The water of hydration was not proved to exist by analysis. Although the analytical results indicated that the salt was not pure, the ratios between the elements showed clearly that a barium salt of a dibasic acid was the major component. The free acid was regenerated by solution of the salt in water and addition of an exact equivalent of  $H_2SO_4$ . The aqueous solution was freed of  $BaSO_4$  and allowed to evaporate slowly in a desiccator over KOH. After many days, crystals were obtained which melted at 119–120°.

$C_{11}H_{11}O_3N_3$ . Calculated. C 41.4, H 5.3, N 13.2  
Found. " 42.0, " 5.1, " 13.0

The crystals were relatively unstable and became oily when stored at room temperature for several months, possibly due to dehydration of the alcoholic group.

This substance as it was obtained in this work was quite probably a mixture of diastereomeric compounds, because, during the hydrolysis, a new asymmetric carbon atom was produced. Since the aspartic acid residue was optically active, the appearance of a second asymmetric center should give a mixture of two isomers, and these may not have been separated during the purification. No special attempt was made to part this mixture.

The question may well be raised as to whether the tertiary alcoholic group actually was present in the propionic acid residue of this substance, or whether it had been eliminated as water, leaving an acrylic acid residue. Although there was no direct evidence on this point, the analytical values agreed better with the postulate that the hydroxyl group remained than with the idea of an acrylic acid compound. The high lycomarasmin activity of the compound (which will be described below) fitted into the belief that the hydroxyl still remained, because such a structure would contain the substituted  $\alpha$ -hydroxyalanine residue which is found in lycomarasmin (2).

*Pyruvylglycylaspartic Acid*—As has already been indicated, this compound was obtained in small yield as an alcohol-insoluble by-product during the preparation of the previously described substance. It was isolated in greater amounts by increasing the rigor of the hydrolysis. Thus, 1 gm. of

with ether and three times with ethyl acetate. The extracts were discarded, and the aqueous phase was concentrated under reduced pressure, and below  $40^{\circ}$ , to a sirup. This was extracted with 500 cc. of acetone, and the soluble portion was concentrated to a thin sirup which was extracted with a few cc. of acetone. This operation caused the separation of a gummy precipitate which was collected and dissolved in 40 cc. of methanol. When chloroform was added to this solution, crystallization began. A yield of 13.8 gm. of hygroscopic, large crystals was obtained.

$C_{11}H_{17}O_7N_3$ . Calculated, N 13.9; found, N 13.9

This compound was prepared as a starting material for the synthesis of pyruvylglycylasparagine, for it was hoped to remove the acetyl group by mild alkaline hydrolysis and then to oxidize the secondary alcohol to a ketone. Although the cleavage of the acetyl group seemed to proceed readily at room temperature in a slight excess of aqueous NaOH, the product obtained was amorphous and very hygroscopic. For these reasons a satisfactory preparation could not be made. Attempted oxidation of the hydrolyzed product with Fenton's reagent for the formation of the pyruvyl compound gave no desired substance which could be purified satisfactorily.

*$\alpha,\alpha$ -Diacetaminopropionylglycylasparagine*—8.5 gm. of the azlactone of  $\alpha,\alpha$ -diacetaminopropionic acid (3) were suspended in 150 cc. of cold alcohol and immediately mixed with an ice-cold solution of 9.5 gm. of glycyl-L-asparagine in 52 cc. of 1 N NaOH. The resulting solution was kept at room temperature for 30 minutes and then acidified with 1 N  $H_2SO_4$  equivalent to the NaOH used, and concentrated under reduced pressure to dryness below  $40^{\circ}$ . The residue was dried by adding 100 cc. of absolute alcohol and again concentrating it under reduced pressure. It was then extracted with 400 cc. of boiling ethanol, and the mixture was filtered hot. A slight precipitate which formed when the filtrate was cooled was discarded, and the alcoholic solution was concentrated under reduced pressure to about 30 cc. and treated with 100 cc. of ethyl acetate. 10 gm. of the product crystallized, and this was recrystallized by adding ethyl acetate to an alcoholic solution. The purified material sintered at  $140^{\circ}$  and melted at  $185$ – $187^{\circ}$ .

$C_{13}H_{21}O_7N_5$ . Calculated. C 43.5, H 5.9, N 19.5  
Found. " 43.5, " 6.2, " 19.0

The neutral equivalent was 370, while the calculated value was 359.

*$\alpha$ -Hydroxy- $\alpha$ -acetaminopropionylglycylaspartic Acid*—1 gm. of diacetaminopropionylglycylasparagine was dissolved in 100 cc. of 0.2 N HCl, and the solution was refluxed for an hour and cooled. It was then adjusted to pH 8 with barium hydroxide, treated with 3 volumes of methanol, and kept in the cold overnight. The precipitate which had formed was collected and



TABLE I

*Responses of Tomato Leaves to Graded Amounts of Lycomarasmin*

Lycomarasmin	Severity of wilting and curling
mg. per 7 cc.	
0.1	0
0.2	+
0.3	++
0.4	+++
0.5	++++
2.0	+++++

TABLE II

*Amounts of Various Compounds Necessary for Wilting and Curling Response in Excised Tomato Leaves*

Compound	Detectable at	Maximum effect at
	mg. per 7 cc.	mg. per 7 cc.
Lycomarasmin.....	0.2	0.8
L-Asparagine.....	5	>30
L-Aspartic acid.....	15	>30
Glycine.....	5	>30
Pyruvylglycine.....	Not detectable at 6	
DL-Serine.....	5	>30
Glycylasparagine.....	15-30	
Glycylaspartic acid.....	Not detectable at 12	
Serylglucylaspartic acid from L-aspartic acid*.....	4-6	8-14
“ “ DL-aspartic “.....	4-6	12
Glycylserylglutamic acid*.....	2-4	6
Glycyl-β-aspartylserine.....	No effect at 14	
Acetylactylglycylasparagine.....	8-12	
Diacetaminopropionylglycylasparagine.....	4	>14
Diacetaminopropionylglycylaspartic acid.....	Not detectable at 15	
α-Hydroxy-α-acetaminopropionylglycylaspartic acid.....	0.3	4
Pyruvylglycylaspartic acid.....	Not detectable at 3	
Serylglucylglutamic acid.....	“ “ “ 7	
Glycylserylglutamic acid.....	“ “ “ 6	
Alanylglucylglutamic acid.....	“ “ “ 7	
Glutathione.....	“ “ “ 6	

\* The activities of these two compounds, which were given in a preliminary note (5), made them appear too active relative to lycomarasmin, because it was found subsequently that the lycomarasmin which had been used for comparison was impure.

wilting than curling in some individual leaves and more curling than wilting in others. To arrive at a value, each leaf was rated on a scale ranging from

$\pm$  to + + + +, by taking into account the extent of wilting, of curling and shriveling, and of obvious necrosis. Between the extremes of  $\pm$  and + + + + there was a range in which graded amounts produced graded severity of change in the leaves. This is illustrated by the data in Table I. The ratings of individual leaves in a group exposed to the same concentration of a substance were usually in good agreement. The values for the activity of a compound could be duplicated by subsequent assays within a factor of 2. Frequently identical values were found in such duplicate determinations.

*Lycomarasmin Activity of Various Compounds*—The lycomarasmin activity of a number of substances was determined in the manner just indicated, and the results are summarized in Table II.

#### DISCUSSION

Among the compounds tested, it is evident that small changes in the nature of the amino acid residues had a greater influence on activity than did slight changes in the position of these residues. In general, even minor changes in the amino acid composition affected potency very much, while considerable alteration in the manner of linkage of the amino acids usually had a minor influence. Lycomarasmin may be viewed as a substance containing 3 amino acid residues; *viz.*, those of asparagine, glycine, and  $\alpha$ -hydroxyalanine. When these three components were maintained, but rearranged so that the  $\alpha$ -hydroxyalanine was attached to glycine in a peptide linkage rather than by a common nitrogen atom (as is the case in lycomarasmin), a relatively active compound,  $\alpha$ -hydroxy- $\alpha$ -acetaminopropionylglycylaspartic acid, was realized, even despite the fact that it was a derivative of aspartic acid rather than of asparagine, and that its amino group was covered with an acetyl radical. However, when the amino acid nature of this substance was changed by moving the hydroxyl to the  $\beta$  position, as in serylglycylaspartic acid, the activity was diminished appreciably. Furthermore, when the change was greater and the amino group eliminated, as in acetylactylglycylasparagine, the resulting compound was much less active than lycomarasmin. The acetyl group in the acetylactylglycylasparagine did not seem to be particularly inimical to potency, because removal of it by mild alkaline hydrolysis did not greatly increase the biological activity. The relative potencies of serylglycylaspartic acid and of serylglycylglutamic acid throw more light on the effect of changing slightly the amino acid composition. The mere introduction of a  $\text{CH}_2$  grouping in passing from aspartic to glutamic acid was enough to reduce the potency of the compound. Other similar examples may be seen from the data in Table II.

In considering what features of the lycomarasmin molecule may be responsible for its biological activity, one should note two points from this

study. The first is that in the three pairs of similar compounds which differed only in that one of the set was an aspartic acid derivative while the other was from asparagine, the asparagine compounds were slightly more active.<sup>1</sup> The second is the rise and fall of activity as one passed, by partial hydrolyses, from diacetaminopropionylglycylasparagine, through  $\alpha$ -hydroxy- $\alpha$ -acetaminopropionylglycylaspartic acid to pyruvylglycylaspartic acid. Maximal potency was displayed in this series by the compound containing an  $\alpha$ -hydroxyalanine residue. It will be remembered that lycomarasmin contained both an asparagine and an  $\alpha$ -hydroxyalanine residue, while inactive Substance I obtained from it by heating, possessed neither.

The effect of changing positions of the amino acid residues may be studied in one of its aspects from the results with the three peptides containing serine, glycine, and aspartic acid. Serylglycylaspartic acid and glycylseryl-aspartic acid were approximately of the same activity. However, glycyl-aspartylserine was inactive. One may argue that the placing of a serine residue on the  $\beta$ -carboxyl group of aspartic acid has blocked the possibility of amidation to asparagine which might take place in the leaves during the test, but nevertheless the inactivity of this position isomer should serve to show the dangers and limitations of generalizations about the relative effects of changing composition compared to altering arrangement of the amino acid residues. Many sorts of isomers must be examined before the question can be answered, even for a simple peptide such as lycomarasmin.

The results with the two preparations of serylglycylaspartic acid, one made from L-aspartic acid and the other from the DL isomer, would tend to suggest that optical configuration is not a crucial matter in lycomarasmin activity. Although the two preparations were admittedly mixtures of diastereomers, which may have been partially separated during the purification procedures, the results were essentially the same when the assays were performed on the crude reaction mixtures of the synthesis, before any purification had taken place.

The large number of substances which showed some ability to wilt and curl tomato leaves must raise a question about the specificity of the test for lycomarasmin. In our experience, almost any substance, even NaCl or  $\text{NH}_4\text{Cl}$ , will cause some wilting of the leaves if the concentration is high enough. This is probably due to disturbances of osmotic pressure, or to other changes, and not to those effects associated with the action of lycomarasmin. Gäumann and Jaag (9) have examined the characteristics of the alterations in leaves attributable to the toxin and have compared them to those found in leaves wilted from other causes. However, at present no way of improving the specificity of the lycomarasmin test seems evident.

<sup>1</sup> Both members of the pair glycylasparagine and glycylaspartic acid were so little active as to make their contribution to this point questionable.

Possibly one qualitative aid might be the extensive and severe character of the changes elicited by the toxin in comparison to those of substances such as glycine or aspartic acid. In these latter cases the production of black, necrotic spots and the drying and shriveling of the leaf edges were not evident as they were with lycomarasmin and with the more active compounds studied in this investigation. The interpretation to be placed on the activities of compounds less than 5 per cent as effective as lycomarasmin must be open to grave doubts. In fact, the biological relationship of any of the substances to the natural toxin will remain open to question until a more specific test can be evolved.

#### SUMMARY

A number of new derivatives of aspartic acid and of asparagine have been prepared in order to examine the effects of various structural changes on the activity of lycomarasmin, which is a peptide derived from asparagine, glycine, and probably  $\alpha$ -hydroxyalanine. Thus, glycylserylaspatic acid, glycyl- $\beta$ -aspartylserine, acetyllactylglycylasparagine,  $\alpha,\alpha$ -diacetaminopropionylglycylasparagine, the corresponding aspartic acid compound,  $\alpha$ -hydroxy- $\alpha$ -acetaminopropionylglycylaspatic acid, and pyruvylglycylaspatic acid were synthesized. These, along with known compounds such as glycylaspatic acid, glycylasparagine, aspartic acid, asparagine, glycine, serine, pyruvylglycine, serylglycylaspatic acid, serylglycylglutamic acid, and glutathione, were compared with lycomarasmin for ability to cause excised tomato leaves to wilt and curl. Several of the peptides were somewhat less active in this respect than was lycomarasmin, but one of them,  $\alpha$ -hydroxy- $\alpha$ -acetaminopropionylglycylaspatic acid, was about equal to the natural toxin in activity. Some produced no detectable effect. Comparison was made of the relative effect on activity of changing the position of an amino acid in a peptide with that of altering the nature of the amino acid. From the limited data, changes in position seemed to affect potency less than replacement by a new amino acid, but the limitations of the argument were recognized. The specificity of the biological test was also discussed.

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# THE INFLUENCE OF PTEROYLGLUTAMIC ACID ON NUCLEIC ACID SYNTHESIS IN *LACTOBACILLUS CASEI*\*

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(Received for publication, July 24, 1948)

Several findings reported in the literature point toward a functional relationship between pteroylglutamic acid (PGA, vitamin B<sub>12</sub>, folic acid) and the synthesis of certain constituents of nucleic acids, but the related specific reactions have not been identified. Snell and Mitchell (1) observed that thymine plus a purine base could partially replace PGA for *Streptococcus faecalis* R in a medium similar to that used by Snell and Peterson (2) in their report on the *Lactobacillus casei* factor. Stokstad (3) observed a similar relationship for *Lactobacillus casei*, but only about one-half maximum acid production could be induced by the substituted nutrient. Stokes (4) suggested that PGA may "function as a coenzyme for the enzyme system responsible for the synthesis of thymine (or more probably a thymine-like compound) which, in turn, is used by the bacteria to form nucleic acid."

Observations by others, however, suggested that PGA is not involved directly in the synthesis of thymine. Hitchings, Falco, and Sherwood (5) studied the effect of pyrimidine derivatives on *Lactobacillus casei*. The organism was inhibited by 5-bromouracil when grown in media containing thymine, but was not inhibited in the presence of PGA. In contrast, 5-nitouracil inhibited growth when PGA was supplied but not when thymine was substituted. This reversal was interpreted as indicating that thymine and folic acid were not components of the same anabolic system. Hall (6) observed a synergistic effect between thymine and PGA in the growth of *Streptococcus faecalis* R and suggested that "thymine may actually be a precursor of folic acid or that thymine is participating in some alternate metabolic path." Strandkov and Wyss (7) observed that an analogue of thymine, thiothymine, competitively inhibited *Lactobacillus casei* in the presence of thymine, but was inactive in the presence of PGA. This was given as "strong evidence that thymine precedes B<sub>12</sub> in some synthetic process." Alternate metabolic pathways for thymine and folic acid could also explain the effect of thiothymine.

Inhibition studies by Lampen and Jones (8, 9) with sulfonamides and by Rogers and Shive (10) with a folic acid analogue, methylfolic acid, indi-

\* This investigation was supported by a grant from the Nutrition Foundation, Inc.

cate that PGA is involved in the biosynthesis of thymine, or a thymine-like compound, and purines. Stokstad *et al.* (11) observed additional interrelationships in cultures of *Streptococcus faecalis* R, indicative that PGA may be involved in the synthesis of thymine and adenine. Stetten and Fox (12) observed, with *Escherichia coli* during bacteriostasis due to sulfonamides, an accumulation of an amine which was identified by Shive *et al.* (13) as 5(4)-amino-4(5)-imidazolecarboxamide. This compound may be either a purine precursor or formed from a purine precursor. Formylfolic acid or rhizopterin has been suggested as a formylating agent in the introduction of a carbon unit into this amine. Schopfer (14) observed that bacteriostasis by sulfathiazole could be offset in part by adenine or by nucleic acids.

In the present investigation, an impairment in the synthesis of desoxyribonucleic acid (DNA), but not of ribonucleic acid (RNA), was found to result regularly from PGA deficiency.

#### EXPERIMENTAL

*Culture and Medium*—The organism used was *Lactobacillus casei* 7469. Monthly transfers were made as stab cultures in a medium containing 1.5 per cent of Bacto-agar, 0.5 per cent of glucose, and 2 per cent of Bacto-yeast extract. After incubation at 37° for 22 hours, the cultures were stored at approximately 5°.

The inoculum was prepared first by a transfer from the stock culture to a tube containing the above medium. After incubation for 18 to 22 hours, a second transfer was made into a medium containing 0.5 per cent of glucose and 2 per cent of Bacto-yeast extract. The cells were centrifuged after 22 hours and washed three times with sterile 0.9 per cent sodium chloride solution. Sterile saline was added to the suspension of cells until a standard density reading was obtained (89 to 90 in an Evelyn photocolormeter, Filter 720). A 2.5 ml. aliquot of this suspension was used to inoculate 500 ml. of the medium described in Table I.

The medium was essentially that of Tepley and Elvehjem (15), with the vitamin modification of Roberts and Snell (16). Half liter quantities were sterilized in 1 liter Erlenmeyer flasks for 15 minutes at 20 pounds pressure. Upon removal from the autoclave, the flasks were cooled in an ice bath.

*Harvesting of Cells*—After incubation at 37° for 22 hours, the inoculated flasks were cooled to 0–5° and centrifuged under refrigeration. The cells were washed once with ice-cold distilled water (volume approximately  $\times 20$ ) and adjusted to volume (100 to 250 ml.).

*Methods of Analysis*—Aliquots of the bacterial cell suspensions were used for the following analyses:

*Dry Weight*—Triplicate samples were dried at 105° for 12 hours.

*Nitrogen*—Duplicate samples were digested according to the method of Koch and McMeekin (17). After appropriate dilution, 1.5 ml. of modified Nessler-Folin reagent (17) were added to 10 ml. of the diluted sample. Light transmission was read in an Evelyn photoelectric colorimeter, Filter 420.

*Acidity*—10 ml. aliquots of the supernatant of the medium were electrometrically titrated with 0.1 N KOH to pH 7.2.

TABLE I  
*Composition of Media*

Component	Quantity per liter	Component	Quantity per liter
Glucose (H <sub>2</sub> O).....	22 gm.	Choline.....	2.5 mg.
Casein*.....	5.0 "	Salts B†.....	5.0 ml.
K <sub>2</sub> HPO <sub>4</sub> .....	2.5 "	<i>p</i> -Aminobenzoic acid.....	100 γ
Sodium acetate (3H <sub>2</sub> O)....	33.2 "	Calcium pantothenate.....	500 "
L-Asparagine.....	100 mg.	Nicotinic acid.....	500 "
DL-Alanine.....	200 "	Pyridoxine hydrochloride...	1000 "
L-Cystine.....	200 "	Thiamine hydrochloride....	500 "
DL-Tryptophan.....	400 "	Biotin‡.....	4 "
Adenine sulfate.....	10 "	Riboflavin§.....	500 "
Guanine hydrochloride....	10 "	Pteroylglutamic acid  .....	2.0 "
Uracil.....	10 "	Thymine¶.....	500 "
Xanthine.....	10 "		

\* Squibb's casein enzymatic hydrolysate, treated four times with 10 per cent by weight of Darco G-60 carbon at pH 3.0 for 0.5 hour periods.

† Salts B = MgSO<sub>4</sub>·7H<sub>2</sub>O 4.0 gm., NaCl 0.2 gm., FeSO<sub>4</sub>·7H<sub>2</sub>O 0.2 gm., MnSO<sub>4</sub>·4H<sub>2</sub>O 0.2 gm., H<sub>2</sub>O to 100 ml., plus 0.5 ml. of 50 per cent H<sub>2</sub>SO<sub>4</sub>.

‡ Biotin-restricted medium contained 0.1 γ per liter.

§ Riboflavin-restricted medium contained 10 γ per liter.

|| Pteroylglutamic acid-restricted medium contained 0.05 γ per liter. We are indebted to Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, for supplying generous quantities of PGA.

¶ Thymine was included only when pteroylglutamic acid was omitted.

*Turbidity*—Transmission was measured in an Evelyn photoelectric colorimeter with Filter 720, adjusted to read 100 when water was used.

*Nucleic Acids*—Duplicate samples were used for the extraction of nucleic acids by the method of Schneider (18). The bacterial cells were treated with 7 per cent trichloroacetic acid in an ice-water bath and stirred every 15 minutes through a 2 hour period. The suspension was then centrifuged under refrigeration and washed once with 20 times its volume of ice-cold 5 per cent trichloroacetic acid. The residue was treated with 40 times its volume of 5 per cent trichloroacetic acid and heated for 15 minutes at 90°, with frequent stirring. After being centrifuged, the residue was re-

heated with 5 per cent trichloroacetic acid for 15 minutes at 90°. The supernatants from the two extractions were combined and made up to volume.

Desoxyribonucleic acid was determined by the diphenylamine reaction described by Dische (19). The desoxyribonucleic acid standard was pre-

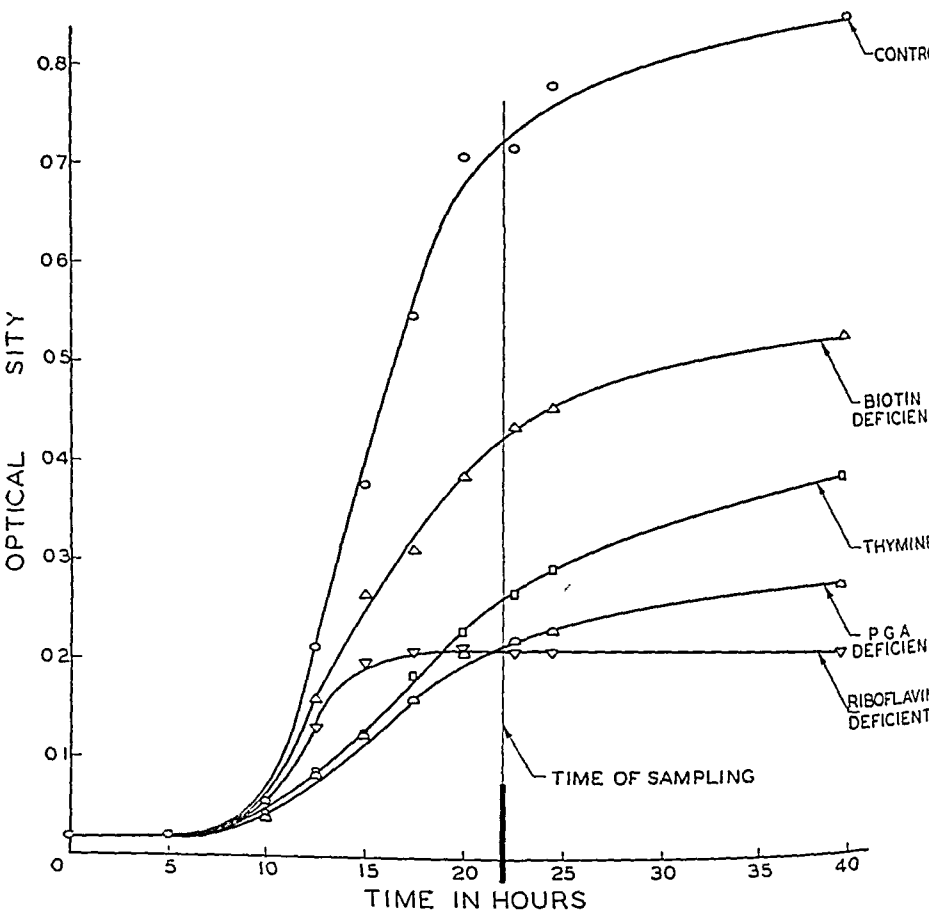


FIG. 1. Rate of growth of *Lactobacillus casei* in media containing excess PGA (control), thymine substituted for PGA, and deficient in PGA, riboflavin, or biotin.

pared from fresh calf thymus glands by the modified method of Levene and Bass (20).

Ribonucleic acid was determined by the Albaum and Umbreit modification (21) of the orcinol reaction (22). A correction (18) for the desoxyribonucleic acid content was applied. The ribonucleic acid standard was a yeast preparation obtained from Merck and Company.



The purity of both standards was estimated from their phosphorus content compared to that calculated for a tetranucleotide structure. On this basis, RNA and DNA were 88.4 and 87.1 per cent pure, respectively. The reported nucleic acid values included consideration of the purity correction applied to the standard nucleic acids. The N:P ratios for RNA and DNA

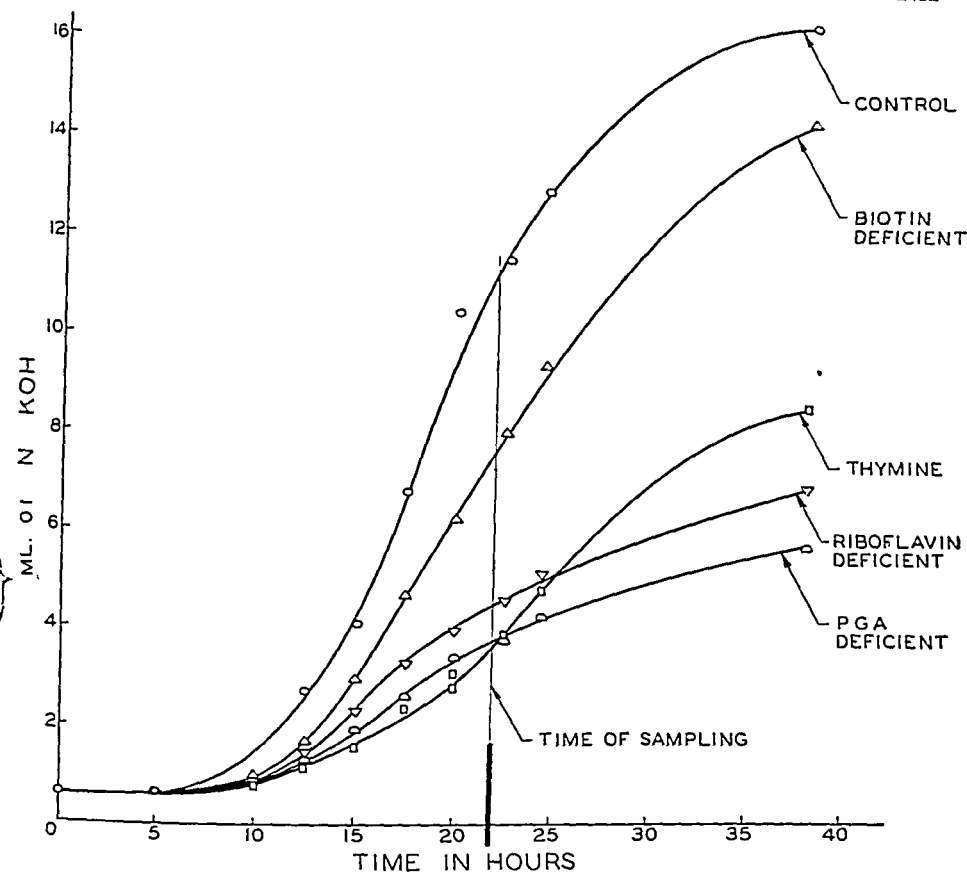


FIG. 2. Rate of acid production of *Lactobacillus casei* in media containing excess PGA (control), thymine substituted for PGA, and deficient in PGA, riboflavin, or biotin.

were 1.68 and 1.60, respectively, compared to the calculated ratios of 1.70 and 1.69. Choice of a proper nucleic acid standard is difficult, because of incomplete information regarding composition of the pure acids.

### Results

*PGA and Thymine Relationships to Nucleic Acid*—A comparison was made of nucleic acid synthesis in media of varying folic acid and thymine

content, bacteria in approximately the same growth phase (22 hours) being used. Data regarding growth and acid production will be noted in Figs. 1 and 2.

As shown in Table II, there was no significant difference in the ribonucleic acid content of bacterial cells when supplied with either abundant or restricted quantities of PGA. There was a significant increase in both types of nucleic acids when thymine replaced PGA in the medium. In contrast, a marked decrease in the desoxyribonucleic acid content resulted when growth was restricted by a PGA deficiency.

TABLE II  
*Nucleic Acids, Nitrogen, Dry Weight, and Acid Production of Lactobacillus casei Grown in Various Media\**

Components varied in media†	Desoxyri- bonucleic acid	Ribonu- cleic acid	Nitrogen	0.1 N acid per 10 ml. media	Total cell mass	0.1 N acid per mg. cells
Series A, averages and ranges of five runs						
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>ml.</i>	<i>gm. per l.</i>	<i>ml.</i>
Control, PGA 2.0 $\gamma$ per liter	1.47 1.22-1.74	12.7 11.6-13.8	8.67 8.00-9.57	10.0 8.2-11.1	1.34 1.08-1.63	0.75 0.68-0.78
PGA-deficient, 0.05 $\gamma$ per liter	0.79 0.56-1.06	12.6 12.1-13.2	9.86 9.19-11.5	3.2 3.1-3.7	0.286 0.220-0.348	1.11 1.06-1.12
Thymine, 500 $\gamma$ per liter	1.89 1.56-2.42	17.3 16.4-17.8	10.4 8.98-12.6	3.0 2.3-3.7	0.302 0.241-0.375	0.98 0.93-1.10
Series B, averages and ranges of four runs						
Control, PGA 2.0 $\gamma$ per liter	1.42 1.33-1.51	13.6 12.9-14.7	9.09 8.00-10.2	10.9 10.8-10.9	1.42 1.41-1.42	0.77 0.76-0.78
Riboflavin-deficient, 10 $\gamma$ per liter	2.08 1.97-2.35	20.0 18.1-22.9	12.1 10.6-12.8	4.1 3.9-4.2	0.212 0.202-0.226	1.93 1.82-2.03
Biotin-deficient, 0.1 $\gamma$ per liter	2.42 2.31-2.50	15.8 14.5-16.8	10.9 9.20-12.0	7.0 6.4-7.6	0.518 0.363-0.690	1.38 1.00-1.76

\* Dry weight basis after 22 hours at 37°.

† Standard components of the media are listed in Table I.

The quantities of acid produced per mg. of dry bacterial cells were almost identical under the conditions of PGA deficiency and when thymine replaced PGA. A higher concentration of PGA caused the acid production to be approximately tripled. Hall (6) observed a decrease in the rate of acid production as the pH of the medium fell, but the change was not marked within the pH range of the tests reported here.

A small increase in the nitrogen content of the bacterial cells was evident both as a result of growth on a PGA-deficient medium and when thymine was substituted for the vitamin.

*Comparison of PGA, Riboflavin, and Biotin Deficiencies*—For comparative purposes, a further study was made of nucleic acid synthesis when growth was restricted by a riboflavin or biotin deficiency. The bacteria when harvested were approaching the end of their active growth period (22 hours) in the medium deficient in biotin. In the riboflavin-restricted medium, growth practically ceased at the end of 15 hours, but acid production continued. The cells were harvested as in the preceding experiment after 22 hours of growth.

The data in Table II show that both the RNA and the DNA contents of bacterial cells subjected to biotin or riboflavin deficiency were significantly higher than in cells that were grown in a PGA-deficient medium or in a medium which contained more nearly optimum concentrations of all nutrients (control medium).

Acid productions in riboflavin- and biotin-deficient media were 38 and 64 per cent, respectively, of the quantities produced under more nearly optimum nutrient conditions. But the amounts of acid produced per mg. of dry cell mass formed in riboflavin- and biotin-deficient media were 250 and 180 per cent, respectively, of the quantities produced under optimum nutrient conditions (Table II).

The nitrogen values on a dry weight basis after growth in riboflavin- and biotin-deficient media were 133 and 120 per cent, respectively, of the control values.

#### DISCUSSION

The degree of PGA restriction in the medium and the time interval for culture growth were selected, in part, on the basis of producing a cell mass and an acidity that would be approximately equivalent to the respective values obtained when excess thymine replaced PGA in the medium. The contrast between desoxyribonucleic synthesis (decrease) and ribonucleic acid synthesis (no change) was sufficiently striking to point toward a specific functional rôle of the nutrient in one or more of the steps essential for synthesizing desoxyribonucleic acid.

In view of the rôle which Boiven *et al.* (23) and Belozersky (24) have suggested for desoxyribonucleic acid in bacterial cells, it would be of interest to explore further the changes that are associated with PGA deficiency. A new type of lead is provided by Shive *et al.* (25), who isolated from hog liver a crystalline compound which was several times as active as folic acid in producing one-half maximum growth and which counteracted methylfolic acid toxicity. This compound was identical with the nucleoside thymidine in x-ray diffraction pattern and biological properties. Hall has reported a product or products derived from histidine by chemical (26) and enzymatic (27) procedures with PGA activity for *Streptococcus lactis* R.

The biotin and riboflavin studies were conducted primarily to find

whether the decreased desoxyribonucleic acid production might be due merely to a non-specific nutrient deficiency. No decrease in the content of the nucleic acids was observed; rather, there was a moderate increase in both cases, but no evidence of a change in proportion of the two products. Price, Miller, and Miller (28) similarly did not find a marked difference in the RNA and DNA storage in the cell nuclei of rats as a result of riboflavin deficiency.

In agreement with observations on other organisms (29, 24, 30) it was found that the nucleic acid content of *Lactobacillus casei* tended to decrease as the age of the culture increased. The RNA content, for example, was 19.5 per cent in a 15 hour culture and 14.7 per cent in the 22 hour culture. The DNA content of a 15 hour culture was 1.83 per cent, and it decreased to 1.33 per cent in the 22 hour culture. This age relationship may explain, in part, the higher nucleic acid values observed with *Lactobacillus casei* grown in riboflavin- or biotin-deficient media and in media with thymine substituted for PGA. These considerations lend emphasis to the observed effects of PGA deficiency.

The effect of thymine in increasing the nucleic acid content above that obtained in the control medium (optimum PGA) does not necessarily support Stokes' (4) hypothesis that folic acid is involved in thymine synthesis, since an increase in both types of nucleic acid also resulted from growth in a medium deficient in either riboflavin or biotin.

Among other observed influences of PGA, failure to form red or white blood cells at a normal rate is one of the most characteristic features of a deficiency in animals. Rodney, Swendseid, and Swanson (31) found that "the rate of tyrosine oxidation by livers from PGA-deficient rats was increased by the addition of PGA." Woodruff and Darby (32) reported that in the scorbutic guinea pig either ascorbic acid or PGA decreased the urinary excretion of tyrosyl derivatives and keto acids, which appeared in abnormally high concentrations due to the addition of L-tyrosine in the diet. Totter and Sims (33) reported that PGA counteracted the inhibitory effect of KCN on both growth and porphyrin production of *Corynebacterium hoffmannii*. Martin and Beiler (34) have reported that 7-methyl-folic acid and the aspartic acid analogue of PGA markedly inhibited dopa decarboxylase in rat kidney preparations. In none of the above cases has there been a basis for citing the specific reactions in which PGA plays a direct rôle.

#### SUMMARY

A partial deficiency of pteroylglutamic acid in a medium otherwise favorable for rapid growth of *Lactobacillus casei* resulted in a distinctly lower content of desoxyribonucleic acid, while the content of ribonucleic acid was essentially unchanged.

A comparable deficiency of riboflavin or biotin did not cause a similar selective effect, but instead resulted in a moderate increase in the content of both types of nucleic acid.

An excess of thymine, adequate to afford growth and acid production rates approximately equal to the respective values resulting from pteroyl-glutamic acid deficiency, caused a moderate rise in both desoxyribonucleic acid and ribonucleic acid.

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# OXYBIOTIN METABOLISM IN THE CHICK\*

## I. DEPOSITION OF OXYBIOTIN IN TISSUES

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(Received for publication, August 5, 1948)

Oxybiotin, the oxygen analogue of biotin, can replace biotin in the nutrition of the rat (1), the chick (2), and certain microorganisms (3). It has been clearly established that *Saccharomyces cerevisiae* and *Rhizobium trifolii* can utilize oxybiotin directly (4, 5). Recently Krueger and Peterson (6) have obtained similar results with *Lactobacillus pentosus*. The present experiments were designed to determine whether oxybiotin is active as such or whether it is converted into biotin by the chick.

If oxybiotin is active as such, its administration to biotin-deficient animals should lead to an accumulation of oxybiotin in the tissues with no concomitant increase in their biotin content. Furthermore, there should be a correlation between the growth response of the animals and the oxybiotin content of tissues. This paper presents the results of a study of the deposition of oxybiotin and biotin in chick tissues following the intramuscular administration of these compounds. By differential assay for biotin and oxybiotin it has been possible to demonstrate that the increased "biological activity" (or biotin-like activity) of tissues (as measured microbiologically) which appears after the administration of oxybiotin to chicks is due to oxybiotin rather than biotin. The growth response of chicks was found to parallel the increase in the oxybiotin content of their tissues.

### EXPERIMENTAL

#### Care of Animals

Day-old white Leghorn cockerels served as experimental animals throughout this study. The basal diet was essentially the same as that used previously for oxybiotin studies (2), and consisted of dextrin, 55.7 per cent; Labco "vitamin-free" casein, 20 per cent; dried raw egg white, 10 per cent; salts, 5 per cent (7); fortified corn oil,<sup>1</sup> 5 per cent; solubilized liver fraction

\* This work was aided by grants from the Buhl Foundation, from the Williams-Waterman Fund of the Research Corporation, and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council. A preliminary report of these studies has appeared (*Federation Proc.*, 6, 276 (1947)).

<sup>1</sup> Per 100 gm. of fortified corn oil:  $\alpha$ -tocopherol 160 mg., 2-methyl-1,4-naphthoquinone 20 mg., vitamin A 80,000 U. S. P. units, and vitamin D 16,000 U. S. P. units.

L,<sup>2</sup> 2 per cent; glycine, 2 per cent; choline chloride, 0.2 per cent; and inositol, 0.1 per cent. To each kilo of ration were added thiamine hydrochloride, 10 mg.; riboflavin, 15 mg.; pyridoxine, 15 mg.; calcium pantothenate, 50 mg.; nicotinic acid, 100 mg.; and pteroylglutamic acid,<sup>3</sup> 1.0 mg. After 1 week on this basal ration, the chicks were separated into four comparable groups on the basis of their weight and growth performance. One group served as a control, while the other three were injected intramuscularly on alternate days with 2, 8, or 20  $\gamma$  of *dl*-oxybiotin (Table I). Two chicks from each group were sacrificed each week for the following 4 weeks, and all of the surviving chicks were sacrificed at the end of the 5th week of injections. Immediately after decapitation, the heart, liver, spleen, and a sample of leg muscle were removed from each chick and kept frozen until analyzed.

A second experiment conducted in a similar manner included the following: (a) a positive control group supplied with a commercial chick ration,<sup>4</sup> (b) two groups injected on alternate days with either 0.3 or 15.0  $\gamma$  of *d*-biotin, (c) two groups injected on alternate days with either 2.0 or 30.0  $\gamma$  of *dl*-oxybiotin, and (d) a negative control group fed the basal ration alone. After 5 weeks of injections, all chicks were sacrificed and tissues were removed for analysis (Table III).

### *Analytical Procedures*

*Preparation of Samples*—Studies were made of various procedures for the extraction of biotin and oxybiotin from tissues. These included autoclaving or refluxing the minced tissue samples for various periods of time up to 6 hours with various concentrations of HCl or H<sub>2</sub>SO<sub>4</sub> up to 6 N. Neither time, concentration of acid, nor choice of acid appeared to be particularly critical in these studies. Autoclaving at 15 pounds pressure for 2 hours with 30 ml. of 5 N HCl per gm. of dry weight of sample was found to liberate the largest amount of "biological activity" from tissues, and this procedure was adopted for general use. Recovery of biotin or oxybiotin added either before or after hydrolysis was good. Following the digestion, each solution was filtered, the residue washed with dilute acid and with water, and the combined filtrates evaporated to dryness. These solids were then dissolved in water, adjusted to pH 6.7 with dilute NaOH, and made up to volume. Aliquots were diluted for microbiological assay.

*Microbiological Assays*—All analyses were made by the Wright and Skeggs (8) microbiological procedure with *Lactobacillus arabinosus* as the

<sup>2</sup> Supplied through the courtesy of Dr. David Klein, The Wilson Laboratories.

<sup>3</sup> Pteroylglutamic acid was kindly supplied by the Lederle Laboratories Division, American Cyanamid Company; all other water-soluble vitamins by Merck and Company.

<sup>4</sup> Purina chick Startena.



test organism.<sup>5</sup> For this organism, 0.4 m $\gamma$  of *dl*-oxybiotin is equivalent to 0.2 m $\gamma$  of *d*-biotin, and the two growth curves are superimposable. The "biological activity" (or biotin-like activity) of a tissue extract, therefore, represents the response to the sum of the biotin and oxybiotin present and is expressed in terms of *d*-biotin.

Two types of differential analysis for biotin and oxybiotin were used. The permanganate procedure (4), modified to use *Lactobacillus arabinosus* as the test organism, and the Raney's nickel procedure (9) were found to give comparable results. In both methods biotin is converted into a compound inactive for *Lactobacillus arabinosus*, by permanganate into the sulfone and by Raney's nickel into desthiobiotin. Any activity remaining after either of these treatments is assumed to be oxybiotin, since under the conditions of these tests oxybiotin is unaffected. Good recoveries of added oxybiotin were obtained. Thus, in a sample containing both biotin and oxybiotin, an aliquot was removed and treated by either permanganate or Raney's nickel and then assayed simultaneously with an untreated aliquot. The difference between the values of the treated and untreated aliquots was a direct measure of the biotin present in the sample.

### Results

The growth response of chicks to graded doses of oxybiotin confirms the earlier observation (2) that *dl*-oxybiotin is approximately 17 per cent as active as *d*-biotin in the nutrition of the chick. Thus, chicks from the second series which received a dosage of 2.0  $\gamma$  of *dl*-oxybiotin attained a greater weight (204 gm.) than those which received 0.3  $\gamma$  of *d*-biotin (175 gm.), while the group receiving 30  $\gamma$  of *dl*-oxybiotin weighed less (222 gm.) than those receiving 15  $\gamma$  of *d*-biotin (268 gm.).

Table I summarizes the "biological activity" found in tissues from chicks injected with graded doses of oxybiotin. No significant variations were observed in the concentrations of biotin-like activity of tissues from chicks sacrificed at the varying time intervals and, therefore, all of the individual figures were included in the averages for each group. Apparently, at each dosage level, the tissue concentrations attained a constant value within 1 week after injections were started. With increasing dosages of oxybiotin a progressive increase in concentration (per gm. of fresh weight of tissue) and in total amount of biotin-like activity per organ was observed. For various tissues the concentrations of biotin-like activity found in the 20  $\gamma$  dosage group were between 5 and 16 times as high as those in the control animals. The total amounts of "biological activity" found in these organs showed even larger increases because of the larger size of the individual

<sup>5</sup> L-Asparagine (0.01 gm.) was added per 100 ml. of the original single-strength medium.

organs in the injected animals. An examination of the relative increases in concentration of biotin-like activity with increased dosages suggests that the tissues approach saturation with a 20  $\gamma$  dosage. A close parallelism was also observed between the growth response and the concentration of biotin-like activity found in individual tissues.

The tissues from the chicks of the first series which received injections of 2  $\gamma$  of *dl*-oxybiotin on alternate days for 5 weeks were subjected to differential assay for oxybiotin and biotin by the Raney's nickel procedure (9) (Table II). Tissues from chicks of the second series which received injections of 30  $\gamma$  of *dl*-oxybiotin on alternate days for 5 weeks were sub-

TABLE I  
"Biological Activity" of Tissues from Chicks Injected with Oxybiotin

Dosage,* $\gamma$ .....	Controls	Oxybiotin-injected chicks		
		2	8	20
Average starting weight,† gm...	55	57	53	54
Average final weight,‡ gm. .	139	201	234	254
Liver, m $\gamma$ per gm.	420 $\pm$ 47§	810 $\pm$ 77	1,900 $\pm$ 70	2,590 $\pm$ 82
" total m $\gamma$	1820 (14)	4840 (14)	12,200 (17)	16,740 (15)
Heart, m $\gamma$ per gm.	34 $\pm$ 5.5	96.5 $\pm$ 9.2	190 $\pm$ 10	220 $\pm$ 7.3
" total m $\gamma$ ..	30 (14)	112 (14)	230 (17)	300 (15)
Spleen, m $\gamma$ per gm.....	23 $\pm$ 3.3	41 $\pm$ 4.1	85 $\pm$ 8.4	120 $\pm$ 4.6
Spleen, total m $\gamma$ .	2.6 (13)	9 (14)	26 (17)	45 (15)
Muscle, m $\gamma$ per gm. ....	12 $\pm$ 1.5 (6)	65.5 $\pm$ 12.5 (6)	120 $\pm$ 5.1 (11)	192 $\pm$ 13 (9)

\* Injected on alternate days into breast muscle.

† After 1 week on basal diet.

‡ Average weight of chicks at end of experiment.

§ Mean  $\pm$  standard error of the mean.

|| The figures in parentheses are the number of tissues analyzed.

jected to differential assay for oxybiotin and biotin by the permanganate procedure (4). Table III summarizes these findings in comparison with the biotin content of tissues from deficient controls, chicks injected with biotin, and chicks on a commercial ration. At these two dosage levels (2 and 30  $\gamma$ , respectively) the concentrations and total quantities of biotin found in tissues from chicks injected with oxybiotin and control chicks were not significantly different. However, despite the similarity of amounts of biotin found in the chicks injected with oxybiotin and control groups, the former groups averaged, respectively, 60 and 83 gm. greater increases in body weight during the 5 week period. Thus, no correlation was observed

TABLE II

*Biotin and Oxybiotin in Tissues from Chicks Injected with Oxybiotin*

	<i>d</i> -Biotin		<i>dl</i> -Oxybiotin	
	<i>total mγ</i>	<i>mγ per gm.</i>	<i>total mγ</i>	<i>mγ per gm.</i>
Liver, injected*	1970 (6)†	300	6420 (6)	980
" control	1820 (14)	420	0	0
Heart, injected	19 (6)	13	150 (6)	110
" control	30 (14)	34	0	0
Muscle, injected	(6)	17	(6)	80
" control	(6)	12	0	0

\* 2 γ of *dl*-oxybiotin on alternate days for 5 weeks.

† The figures in parentheses are the number of tissues analyzed.

TABLE III

*Comparison of Biotin and Oxybiotin Content of Tissues from Various Chick Groups*

Groups	Deficient controls	Oxybiotin-injected*		Biotin-injected†	Commercial diet
	<i>d</i> -Biotin	<i>d</i> -Biotin	<i>dl</i> -Oxybiotin	<i>d</i> -Biotin	<i>d</i> -Biotin
Liver, <i>mγ per gm.</i>	440 ± 75‡	356 ± 41	4,250 ± 210	3,370 ± 250	2,360 ± 60
Liver, <i>total mγ.</i>	2020 (8)§	2240 (10)	31,530	28,000 (7)	27,000 (8)
Heart, <i>mγ per gm.</i>	38 ± 10	34 ± 3	396 ± 28	263 ± 12	274 ± 15
Heart, <i>total mγ.</i>	36 (8)	36 (10)	430	356 (7)	488 (8)
Muscle, <i>mγ per gm.</i>	11 ± 1 (5)	4 ± 1 (5)	94 ± 4	61 ± 4 (5)	
Spleen, <i>mγ per gm.</i>	9 ± 1	4.3 ± 2	124 ± 5	60 ± 8	
Spleen, <i>total mγ.</i>	1.6 (5)	2.5 (5)	63	28 (5)	
Lung, <i>mγ per gm.</i>		3.2 ± 1	67 ± 4	35 ± 2	52
Lung, <i>total mγ.</i>		4.3 (8)	89	51 (8)	93 (3)
Starting weight, <i>gm.</i>	52		53	53	64
Final weight, <i>gm.</i>	138		222	268	412

\* 30 γ of *dl*-oxybiotin on alternate days for 5 weeks.† 15 γ of *d*-biotin on alternate days for 5 weeks.

‡ Mean ± standard error of the mean.

§ The figures in parentheses are the number of tissues analyzed.

|| After 1 week on a basal or commercial diet.

between the growth response and actual biotin content of tissues. In contrast, the growth response was found to increase with increasing tissue concentrations of oxybiotin.

When microbiologically equivalent dosages of *d*-biotin (15  $\gamma$ ) and *dl*-oxybiotin (30  $\gamma$ ) were injected into chicks, the concentrations of biotin-like activity found in chick tissues (by microbiological assay) were quite similar (Table IV). Since the increase in biological activity resulting from the injection of *dl*-oxybiotin has been shown to be due to oxybiotin, it is apparent that the storage of oxybiotin and biotin was approximately the same at these injection levels. However, the group injected with biotin

TABLE IV  
"Biological Activity" of Tissues from Chicks Injected with Biotin or Oxybiotin

	Biotin-injected*	Oxybiotin-injected†
	my per gm.	my per gm.
Liver.....	3370	2480
Heart.....	263	232
Muscle.....	61	51
Spleen.....	60	66
Lung.....	35	36

\* 15  $\gamma$  of *d*-biotin on alternate days for 5 weeks.

† 30  $\gamma$  of *dl*-oxybiotin on alternate days for 5 weeks.

TABLE V  
"Biological Activity" Liberated by Autoclaving in Acid or in Water\*

		Liver	Heart	Muscle	Spleen
Autoclaved 2 hrs. in 5 N HCl	Average	2930	293	158	131
	Range	2220-3460	246-393	129-214	88-205
Autoclaved 2 hrs. in distilled H <sub>2</sub> O	Average	172	35	35	34
	Range	98-223	22-60	24-41	17-56
% liberated in distilled H <sub>2</sub> O	Average†	5.7	12.6	22	28
	Range	4.4-8.0	6.0-24	15-29	14-44

\* Values from six chicks which received 20  $\gamma$  of *dl*-oxybiotin on alternate days for 5 weeks. The values are expressed as my per gm. of fresh tissue.

† On assumption of 100 per cent liberation by 5 N HCl.

on the average gained 45 gm. more than the oxybiotin group. The differences in growth performance, therefore, are not the result of differences in absorption and storage, but the direct result of intrinsic variations in the biological activities of the two molecules in the chick.

Since a large percentage of the biotin of animal tissues is present in a bound form, that is, requires hydrolysis with acid or enzymes for its liberation, it was of interest to determine whether the "biological activity" present in animal tissues following the injection of oxybiotin was similarly bound. Tissues from chicks which had received 20  $\gamma$  of *dl*-oxybiotin on

alternate days for 5 weeks were used in the experiment. Each tissue was divided into approximately equal portions and these were autoclaved for 2 hours in either 5 N HCl or in distilled water. Microbiological assays were conducted on the filtrates. The findings are summarized in Table V. It is apparent that only a small proportion of the "biological activity" was liberated by autoclaving in water. Thus, the "biological activity" stored following the injection of oxybiotin was bound in a manner comparable to that of biotin.

#### DISCUSSION

The storage of oxybiotin in chick tissues has been repeatedly observed during these studies. This is in marked contrast with the report by Moore, Luckey, Elvehjem, and Hart (10) that "no appreciable accumulation" occurred in either liver or muscle of the chick until 1000  $\gamma$  of *dl*-O-heterobiotin (oxybiotin) was fed per 100 gm. of diet. Although no food consumption figures were reported, it is obvious that the intake of their animals was many times the 1  $\gamma$  per day which in our experiments induced a large deposition of oxybiotin in muscle, heart, and liver tissue. After only 1 week of injections, sufficient oxybiotin had been deposited in liver and muscle to more than double the "biological activity," compared to deficient control tissues. Apparently the injection of small quantities of oxybiotin leads to a greater and more prompt deposition in tissues than the feeding of high concentrations in the ration. It should be noted that the differential assay employed by Moore *et al.* depends on the difference in response of *Streptococcus faecalis* R to biotin and oxybiotin. In our hands this method has not proved satisfactory.

Since biotin-deficient chicks show a definite growth response to injections of oxybiotin without any increase in the biotin content of various body tissues, and since the amount of the growth increase parallels both the amount of oxybiotin injected and the amount stored in tissues, it seems highly probable that oxybiotin is biologically active as such. The similarity of binding in tissues is in agreement with this concept. Unequivocal proof that oxybiotin is not converted into biotin in the chick is provided by complete balance experiments which are presented in the following paper.

#### SUMMARY

1. Liver, heart, spleen, lung, and leg muscle from chicks which received oxybiotin injections into the breast muscle were found to have a high content of oxybiotin.
2. The actual biotin content of these tissues was almost identical with that of tissues from biotin-deficient chicks.
3. The growth response of chicks injected with oxybiotin paralleled the

quantities of oxybiotin injected and the amounts of oxybiotin found in tissues.

4. When microbiologically equivalent amounts of oxybiotin and biotin were injected into similar groups of chicks, comparable amounts of both compounds were stored in chick tissues.

5. Oxybiotin, like biotin, was only partially liberated from tissues by autoclaving in distilled water, yet readily liberated by autoclaving in 5 N HCl.

These findings are in agreement with the concept that the biological activity of oxybiotin is an intrinsic property of the molecule rather than a result of its conversion into biotin.

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# OXYBIOTIN METABOLISM IN THE CHICK\*

## II. OXYBIOTIN AND BIOTIN BALANCE STUDIES

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Balance experiments have demonstrated clearly that oxybiotin is not converted into biotin during the growth of *Saccharomyces cerevisiae* 139, *Rhizobium trifolii* (1), or *Lactobacillus pentosus* 124-2 (2). Growth experiments with chicks, together with studies of the accumulation of oxybiotin in their tissues, suggest that no conversion occurs in the chick (3). This paper presents complete balance experiments demonstrating that the chick does not convert oxybiotin into biotin and, therefore, that the biological activity of oxybiotin is an intrinsic property of the molecule.

### EXPERIMENTAL

In the first series, ten newly hatched white Leghorn cockerels of identical weights were placed in pairs in screen bottom cages over glass funnels. The excreta were washed down daily from the cages and funnels into flasks containing 2 cc. of concentrated HCl and toluene and these collections were kept in the refrigerator until analyzed. Basal diet and water were supplied *ad libitum*. Daily records of chick weights and food consumption were kept. The diet consisted of sucrose, 60.2 per cent; Labco "vitamin-free" casein, 25 per cent; salts, 5 per cent (4); fortified corn oil, 5 per cent;<sup>1</sup> solubilized liver fraction L, 2 per cent;<sup>2</sup> glycine, 2 per cent; L-cystine, 0.3 per cent; L-arginine, 0.2 per cent; choline chloride, 0.2 per cent; and *i*-inositol, 0.1 per cent. The vitamin supplement was identical with that used in previous experiments (3). After 1 week, the chicks were separated into two comparable groups. One group of six chicks served as the negative control; the other group of four was injected intramuscularly on alternate days with eight equal dosages totaling 32  $\gamma$  of *dl*-oxybiotin.

\* This work was aided by grants from the Buhl Foundation, from the Williams-Waterman Fund of the Research Corporation, and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council. A preliminary report of these studies has appeared (*Federation Proc.*, 6, 276 (1947)).

<sup>1</sup> Contained as follows per 100 gm. of fortified corn oil:  $\alpha$ -tocopherol 160 mg., 2-methyl-1,4-naphthoquinone 20 mg., vitamin A 80,000 U. S. P. units, and vitamin D 16,000 U. S. P. units.

<sup>2</sup> Supplied through the courtesy of Dr. David Klein, The Wilson Laboratories.

The animals in the second series were treated in like manner except that they were placed in individual cages. This series included three equal groups (based on their weight and growth performance) of four chicks each. One group was the negative control; a second group received 10.2  $\gamma$  of *d*-biotin; the third group received 68  $\gamma$  of *dl*-oxybiotin. At 3 weeks of age the chicks of both series were sacrificed (chloroform) and their carcasses frozen. For analysis, these frozen carcasses were chopped into small pieces and the entire chick hydrolyzed.

For comparison, biotin determinations were made on twelve newly hatched chicks comparable with those used in the experimental groups. These were found to have a biotin content of  $5.1 \pm 0.3$   $\gamma$  per chick (mean  $\pm$  standard error).

Digestions of the chicks, the excreta, and the food samples were carried out with 5 N HCl in the manner described in the previous paper (3). Differential microbiological assays for biotin and oxybiotin were made by both the permanganate (1) and the Raney's nickel methods (5), with *Lactobacillus arabinosus* as the test organism. Added biotin was completely destroyed. Good recoveries of oxybiotin were obtained.

### Results

Table I summarizes the *biotin* balance studies of the individual chicks used in these experiments. The excreta samples contained approximately the same amounts of biotin for successive weeks with no increase following the injection of oxybiotin. For simplicity, only the figures for the total excretion of biotin during the 3 week period are presented. Both the permanganate and Raney's nickel procedures were used for differential analysis and both sets of figures are presented. (These analyses were performed independently in two different laboratories.) Considering the many steps involved, the results obtained with both procedures in general are in close agreement. The Raney's nickel method, however, gave somewhat higher results for the biotin content of the excreta, although added biotin was quantitatively destroyed. However, even with the Raney's nickel figures for the total biotin content of each chick plus excreta, the chicks injected with oxybiotin did not have a significantly higher content of biotin than non-injected controls (8.6  $\gamma$  total for chicks injected with oxybiotin compared to 7.6  $\gamma$  for non-injected controls). With the permanganate method lower quantities of biotin were found (7.1  $\gamma$ ). The biotin content of chicks injected with oxybiotin was identical with that of the controls and not significantly different from that of newly hatched chicks (5.8  $\gamma$  in non-injected controls, 5.4  $\gamma$  by permanganate and 6.0  $\gamma$  by Raney's nickel in chicks injected with oxybiotin, and 5.1  $\gamma$  in comparable day-old chicks).

In the first series the total amount of basal diet consumed contained from



0.37 to 0.55  $\gamma$  of biotin per chick, while in the second series (supposedly identical, but made up of a different batch of ingredients) the food provided

TABLE I  
*Biotin Content of Individual Chicks and Excreta*

dl-Oxybiotin-injected animals							Control animals			d-Biotin-injected animals			
Dosage	Permanganate procedure			Raney's nickel procedure						Dosage	Chick	Excreta	Total
	Chick	Excreta	Total	Chick	Excreta	Total							
$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	
32	5.3	3.3	6.9*	5.0	3.6	6.8*	4.7	3.3	6.3*	10.2	9.8	2.9	12.7
32	5.1		6.8	5.0		6.8	4.3		6.0	10.2	11.5	2.7	14.2
32	4.3	2.2	5.4*	4.5	4.2	6.6*	5.0	2.9	6.5*	10.2	15.8	3.5	19.3
32	7.1		8.2	8.1		10.2	5.8		7.2	10.2	11.5	3.9	15.4
68	5.1	2.1	7.2	7.3	4.4	11.7	9.5	3.3	11.1*				
68	3.9	2.5	6.4	5.5	3.9	9.4	5.8		7.5				
68	7.5	1.8	9.3	7.8	2.6	10.4	5.0	1.8	6.8				
68	5.0	1.3	6.3	4.5	2.6	7.1	8.5	2.1	10.6				
							5.1	1.8	6.9				
							4.4	2.4	6.8				
Average...	5.4	1.7	7.1	6.0	2.7	8.6	5.8	1.8	7.6		12.2	3.3	15.4

\* Assuming equal amounts of biotin were excreted by each chick in the pair.

TABLE II  
*Oxybiotin Content of Individual Chicks and Excreta*

dl-Oxybiotin injected	Permanganate procedure				Raney's nickel procedure			
	Chick	Excreta	Total	Recovery	Chick	Excreta	Total	Recovery
$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	$\gamma$	per cent
32	15.3	22.3	26.5*	83	13.8	20.3	24.0*	75
32	18.8		30.0	94	17.5		27.7	87
32	13.5	21.0	24.0*	75	10.0	16.5	18.3*	57
32	15.6		26.1	81	13.8		22.1	69
68	25.8	22.0	47.8	70	25.3	17.7	43.0	63
68	32.3	20.4	52.7	77	33.0	17.3	50.3	74
68	25.0	28.1	53.1	78	26.8	21.6	48.4	71
68	26.0	34.2	60.2	88	24.9	28.6	53.5	79

\* Assuming equal amounts of oxybiotin were excreted by each chick in the pair.

from 1.2 to 2.0  $\gamma$ . The excretion by the non-injected control chicks of amounts of biotin greater than that present in the food consumed suggests that bacterial synthesis of small amounts of biotin may have occurred.

Analysis of comparable chicks which received biotin injections indicated that a large amount of the biotin could be recovered in the chick plus excreta (see Table I). Thus, if appreciable quantities of biotin were formed from oxybiotin in chicks injected with oxybiotin, it should have been detectable. The failure to find any increase in the total biotin of the chick or excreta following the injection of large dosages of oxybiotin excludes the possibility of oxybiotin being converted into biotin by the chick.

In Table II are presented the recoveries of oxybiotin injected into chicks. With a dosage of 32  $\gamma$  of *dl*-oxybiotin 31 to 59 per cent was recovered within the chick, while 30 to 35 per cent was recovered in the excreta. 75 per cent or more of the injected oxybiotin was accounted for in the chick plus excreta. With the higher dosage of 68  $\gamma$  of oxybiotin, 37 to 48 per cent was recovered within the chick and 25 to 50 per cent in the excreta. Certainly the recovery of oxybiotin was very good under the conditions of these experiments. The high recovery of oxybiotin precludes the possibility of its being converted into biotin.

#### DISCUSSION

The biotin balance experiments described in this paper demonstrate clearly that the chick does not convert oxybiotin into biotin. This conclusion is further supported by the fact that about 75 per cent of the injected oxybiotin could be accounted for unchanged in the chick plus excreta. Recovery of injected biotin was of the same order.

It must be concluded that the increased growth consistently observed following the administration of oxybiotin to biotin-deficient chicks is the result of the direct utilization of this compound. The experiments reported here complete the evidence for the first demonstration that the sulfur atom of biotin is not essential for the biological activity of this compound in a higher animal. Since several widely different species can utilize oxybiotin as such, it seems reasonable to predict that oxybiotin can replace biotin in all biological forms.

#### SUMMARY

1. The biotin content of chicks injected with oxybiotin was identical with that of control chicks and not significantly different from that of newly hatched chicks.
2. The biotin excretion by chicks injected with oxybiotin was identical with that of control animals.
3. When total dosages of 32 or 68  $\gamma$  of *dl*-oxybiotin were injected into chicks over a 2 week period, approximately 75 per cent of the oxybiotin was accounted for almost equally distributed between the chick and excreta. A similar recovery of injected biotin was observed.

4. Since the chick does not convert oxybiotin into biotin, the biological activity of this compound must be an inherent property of the molecule.

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# SYNTHESIS OF CARBONYL-LABELED PYRUVIC ACID\*

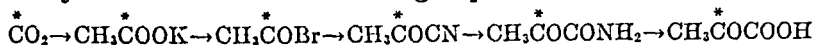
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(Received for publication, July 12, 1948)

Carbonyl-labeled pyruvic acid was synthesized from radioactive carbon dioxide for use in feeding experiments, the results of which are reported in the following communication (1).

The synthesis involves the following steps.



*1-C<sup>14</sup> Potassium Acetate*—The method described here does not differ in principle from those published earlier (2). The apparatus used is illustrated in Fig. 1. 25 mm of radioactive barium carbonate are introduced into the generator, 50 ml. of hydrochloric acid-water, 1:10, into the dropping funnel, and a saturated barium hydroxide solution into the last trap. After a moderate stream of nitrogen has been started, 100 ml. of an ethereal 0.5 N methyl magnesium bromide solution<sup>1</sup> are pipetted into the reaction vessel, which is cooled in an ice bath. After all the air has been displaced, 100 ml. of 0.1 N sodium hydroxide solution are added to the gas absorption bottle.<sup>2</sup> The nitrogen stream is reduced and the hydrochloric acid added rapidly to the barium carbonate. The reaction vessel is continuously shaken. When the barium carbonate is decomposed, the nitrogen stream is increased again. The reaction product precipitates as the carbon dioxide enters the reaction vessel. After shaking for 20 to 30 minutes, 50 ml. of ice water are introduced into the reaction vessel. When the precipitate has dissolved, 30 ml. of 2 N sulfuric acid are added and the ice bath removed. All additions are made after pressure equalization and without interruption of the nitrogen stream, which is continued until the ether layer has evaporated completely. 60 mm of silver sulfate and 50 ml. of concentrated sulfuric acid are added and the mixture is distilled with steam in an all-glass distilling apparatus. The distillate is neutralized with 0.1 N potassium hydroxide and concentrated to dryness. The residue is dissolved in 100 ml. hot methanol and filtered after addition of a small quantity of charcoal. The filtrate is again evaporated and dried. The yield is 65 to 85 per cent, based on the barium carbonate used. Nearly all unchanged carbon dioxide

\* This work was supported in part by a grant from the United States Public Health Service.

<sup>1</sup> Arapahoe Chemicals, Boulder, Colorado.

<sup>2</sup> Barium hydroxide solution is unsuitable here, as precipitated barium carbonate may clog the fritted disk.

can be recovered from the alkali trap. (Only a very small quantity of barium carbonate should have precipitated in the barium hydroxide trap.)

*1-C<sup>14</sup> Acetyl Bromide (3)*—20 mm of potassium acetate are pulverized, mixed with 16 mm of benzoic acid, and added to a distilling flask. Two portions of 2 mm each of benzoic acid are used to "wash" out the flask which contained the potassium acetate. 10 ml. of benzoyl bromide are added to the mixture, a glass wool plug is inserted below the side-arm, and the flask heated carefully so that the acetyl bromide distils slowly into the tared receiving flask. The boiling range is 72–76°. Yield, 75 to 90 per cent.

*1-C<sup>14</sup> Acetyl Cyanide (4)*—8 mm of acetyl bromide are added to 9 mm of dry cuprous cyanide contained in an ice-cooled ampul. Sufficient dry cyclohexane to wet all the cuprous cyanide is added and the ampul is sealed

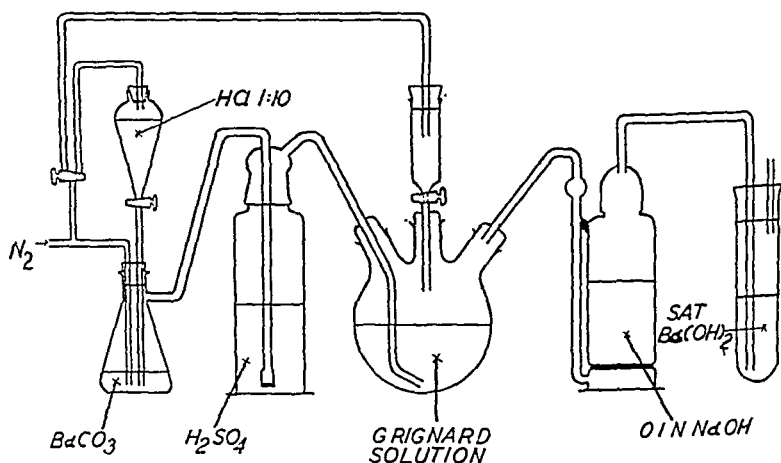


FIG. 1. Apparatus for the synthesis of labeled acetic acid. (The wash bottles are Corning catalogue, Nos. 31770 and 31750.)

off. After standing for 3 days at room temperature the contents are extracted with five portions of 2.5 ml. each of dry ether. The ether solution is used directly. Yield, 75 to 80 per cent.

*2-C<sup>14</sup> Pyruvamide (5)*—The ether solution is introduced into a jacketed funnel with a fritted filter disk through which dry hydrogen chloride gas passes from the bottom. Ice water is circulated through the jacket and a calcium chloride tube is attached at the top. After the solution is saturated with hydrogen chloride, 6.5 mm of water are added to the ether solution and the hydrogen chloride stream continued for 30 minutes. Pyruvamide crystallizes out. The hydrogen chloride stream is discontinued and the material filtered by gravity. The precipitate is washed twice with 5 ml. each of ether-cyclohexane, 5:1, saturated with hydrogen chloride gas. The

top of the funnel is immediately connected to a nitrogen stream in order to drive off the hydrogen chloride completely (about 3 hours). The pyruvamide obtained is recrystallized from a minimum amount of dry ethyl acetate. Yield, 40 to 70 per cent; m.p. 127°.

*2-C<sup>14</sup> Pyruvic Acid*—A solution of pyruvic acid is obtained by dissolving 1 mm of pyruvamide in 1 to 3 ml. of water, adding 1 ml. of 1 N hydrochloric acid, and heating on a steam bath for 90 minutes. The pyruvic acid content is determined colorimetrically (6). In view of the known instability of pyruvic acid, the solution should be used as soon as possible. Yield, 90 to 100 per cent.

#### SUMMARY

Pyruvic acid labeled with isotopic carbon in the carbonyl group was synthesized from isotopic carbon dioxide as starting material in about 40 per cent over-all yield. Potassium acetate, acetyl bromide, acetyl cyanide, and pyruvamide were obtained as intermediates in the synthesis.

The product was stored in the form of pyruvamide rather than pyruvic acid in view of the stability of this compound.

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# SOME ASPECTS OF THE METABOLISM OF PYRUVIC ACID IN THE INTACT ANIMAL\*

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(Received for publication, July 12, 1948)

Until the advent of the isotope technique, the formation of fat in adult animals was held to occur only on a diet containing an excess of calories. From the work of Schoenheimer and Rittenberg (1) it became clear that body fat is not an inert energy store but participates actively in intermediary metabolism. Their evidence also indicated that small molecules were utilized to build up the fatty acid chains as well as the cholesterol structure. The observation that carbohydrate can serve as a precursor for body fat is very old, and many investigations have been carried out to analyze the details of this process. It is clear that on a balance basis fat must ultimately be derived from the carbohydrate or protein of the diet if no fat is supplied in the food. Stetten and Boxer (2) concluded from experiments with heavy water that a large portion of dietary carbohydrate is metabolized by way of fat. These authors (3) proposed too that interference with fatty acid synthesis is one of the main metabolic defects in alloxan diabetes. Investigations by Bloch and Rittenberg (4, 5) revealed the importance of acetic acid as a precursor in the synthesis of fatty acids and cholesterol and established the quantitative significance of acetic acid as a major intermediate of metabolism.

Smedley and Lubrzenska (6) first suggested pyruvic acid as an important intermediate in carbohydrate metabolism, and many investigations since have borne out the central rôle of pyruvic acid in glycolysis. It seems likely that pyruvic acid is also a key intermediate in the conversion of carbohydrate to other body constituents. The work presented here was undertaken to ascertain directly the utilization of pyruvic acid for the various metabolic processes, particularly the synthesis of acetic acid, fatty acids, and cholesterol. For this purpose pyruvic acid labeled in the the carbonyl carbon with  $C^{14}$  was synthesized and fed to rats. Feeding experiments were also carried out with carboxyl-labeled pyruvic acid and labeled acetic acid.

## EXPERIMENTAL

*Syntheses of 1- $C^{14}$  Acetic Acid and 2- $C^{14}$  Pyruvic Acid*—These preparations are described in the preceding communication (7).

\* This work was supported by a grant from the United States Public Health Service.

*Synthesis of 1-C<sup>13</sup> Pyruvic Acid*—The synthesis was carried out in the same manner as described for 2-C<sup>14</sup> pyruvic acid, except that normal acetyl bromide and labeled cuprous cyanide were used as starting materials. Labeled cuprous cyanide was prepared by shaking equimolar amounts of solid cuprous chloride and sodium cyanide in an aqueous solution.

*Feeding Experiments*—Growing rats weighing about 120 gm. were fed *ad libitum* for 3 days on a diet consisting of 73 per cent corn-starch, 16 per cent casein, 5 per cent dried yeast, 4 per cent salt mixture (8) and 2 per cent cod liver oil. The pyruvic acid hydrolysate or sodium acetate dissolved in water was mixed with the diet. 100 mg. of *p*-aminobenzoic acid per 100 gm. of body weight or 50 mg. of  $\gamma$ -phenylaminobutyric acid per 100 gm. of body weight were added to the diet when desired. Two strains of rats, a mixed laboratory strain whose original stock consisted of Wistar rats, and the Sprague-Dawley strain, were used in these experiments. In all cases the experimental conditions were kept as closely alike as possible.

### *Isolation of Body Constituents*

*Acetyl Derivatives*—Acetyl-*p*-aminobenzoic acid and acetyl- $\gamma$ -phenylaminobutyric acid were isolated from the urine and purified as described by Bloch and Rittenberg (9).

*Hippuric Acid and Glycine*—Hippuric acid was obtained from the urine and glycine isolated after hydrolysis of the hippuric acid, according to the method of Shemin (10).

*Liver Glycogen*—The animals were killed by exsanguination in ether narcosis. The livers were immediately dispersed in ethanol in a Waring blender and digested after the addition of 1 volume of 20 per cent KOH by heating for 2 hours. The insoluble glycogen was centrifuged and purified according to the procedure of Stetten and Boxer (2).

*Cholesterol and Fatty Acids*—Cholesterol was precipitated as the digitonide from the unsaponifiable fraction. The digitonides obtained were decomposed with pyridine (11) and the free cholesterol recovered. The saturated fatty acids were separated via the lead soaps according to the procedure of Schoenheimer and Rittenberg (12).

*Urea*—After extraction of the excreted acetyl derivatives, the urine was adjusted to pH 5 and treated with urease. The carbon dioxide liberated was precipitated as barium carbonate.

*Decarboxylation*—Fatty acid was heated together with powdered iron to 300° in a nitrogen stream. Carbon dioxide was precipitated as barium carbonate, and the ketones obtained from the residue recrystallized from ethanol (13).

*Isotope Analyses*—The isolated compounds were burned in a micro combustion apparatus to carbon dioxide. The combustions were carried out at about 900° with a Vicor combustion tube and a platinum gauze filling. The carbon dioxide was precipitated as barium carbonate.

For analysis of  $C^{14}$  the barium carbonate was suspended in methanol, the suspension transferred to cups, and a layer of barium carbonate 8 to 15 mg. per sq. cm. thick was deposited. The samples were counted with a thin window Geiger-Muller counter for a sufficient length of time to give less than 5 per cent probable error. The number of counts was corrected for activity of infinitely thick samples with the curve published by Reid (14). The size of the cups (area, 3.47 sq. cm.) and the counting procedure were identical for all analyses.

For analysis of  $C^{13}$  the barium carbonate was converted to carbon dioxide in a vacuum system (15) and the gas analyzed in a mass spectrometer.<sup>1</sup> The analyses have an error of about  $\pm 0.01$  per cent excess  $C^{13}$ .

Throughout this communication the term "relative isotope concentration" (*RIC*) is used for reporting the analytical data. It is calculated in the following manner.

$$RIC = \frac{\text{radioactivity of isolated compound}}{\text{radioactivity of fed compound}} = \frac{\text{atom \% excess } C^{13} \text{ in isolated compound}}{\text{atom \% excess } C^{13} \text{ in fed compound}}$$

The "relative isotope concentration" is independent of the kind of isotope used for individual experiments; *i.e.*, the same figure is obtained if an experiment is carried out with either  $C^{13}$  or  $C^{14}$ . Furthermore, by reporting the data as "relative isotope concentration," the analytical values of the isolated material become independent of the isotope concentration of the administered substance.

In the experiments reported here the weight of the animals as well as the amounts of the labeled test substance varied. For comparison of the analytical data from different experiments the term "concentration coefficient"<sup>2</sup> (*CC*) is introduced.

<sup>1</sup> The construction of the mass spectrometer was made possible by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and from the Charles H. and Mary F. S. Worcester Memorial Fund.

<sup>2</sup> The concentration coefficient is calculated from the isotope dilution formula (16),  $((C_q/C) - 1)Q = ((C_q/C') - 1)q$ , where  $C_q$ ,  $C_q$  are the isotope concentrations of fed material,  $C$  is the isotope concentration of isolated material after feeding  $Q$ ,  $Q$  is the quantity of fed material (in mμ per 100 gm. per day),  $C'$  is the isotope concentration calculated if  $q$  would have been fed, and  $q$  is the standard quantity. If the relative isotope concentrations are used and if the standard quantity is 1 mμ per 100 gm. per day, then  $C_q = C_q = 1$ ,  $q = 1$ ,  $C = RIC$ , and  $C' = CC$ . After substitution of these values and solving for  $CC$ , the formula for the concentration coefficient is obtained. In most cases the expression  $(1 - Q)RIC$  in the denominator is negligible and the actual calculation can be simplified accordingly. The "concentration coefficients"

$$CC = \frac{RIC}{Q + (1 - Q)RIC}$$

where  $Q$  is the quantity of the compound fed in mm per 100 gm. of rat tissue per day.

The "concentration coefficient" affords the "relative isotope concentration" which would have been found if exactly 1 mm per 100 gm. per day had been fed.

In cases in which the administered compounds contain several carbon atoms of which only one is labeled, the concentration coefficient may not be a true measure of the utilization of the test substance. The possibility exists that not all carbon atoms of the fed material are incorporated to an equal extent into the isolated compounds. In such a case the "specific concentration coefficient"<sup>2</sup> ( $SCC$ ) relating only to the carbon atoms utilized will be of greater significance.

$$SCC = CC \times f$$

where  $f$  is the fraction of carbon atoms of fed compound utilized.

In Tables I, II, and V the "specific concentration coefficient" is given only in those instances in which the mechanism could be established experimentally, such as the utilization of carbon atoms 2 and 3 of pyruvic acid only for the acetyl groups and the fatty acids.

### Results

The analytical data are given in Tables I to VI. The isotope concentrations found indicate that pyruvic acid is incorporated to a varying degree into the acetyl groups of the excreted foreign amines, liver glycogen, cholesterol, and fatty acids.

In order to evaluate the extent to which a labeled precursor is utilized for the synthesis of a body constituent, it is necessary to know the isotope concentration of the precursor at the site of synthesis. This value depends on the dilution of the administered precursor by its endogenous analogue, *i.e.* the size of the metabolic pool. In the case of pyruvic acid it was not possible to determine the size of the pool directly. However, an indirect estimate was obtained in view of the fact that pyruvic acid provides acetyl groups for the acetylation of some foreign amines.

*Acetyl Groups*—It is possible to determine the isotope concentration of the endogenous acetyl pool of the liver by taking advantage of the acetyla-

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defined here are similar to the "coefficients of utilization" of Bloch and Rittenberg (17). The "coefficients of utilization" were used to describe the efficiency of several test substances in forming the same metabolite, while the "concentration coefficients" are used to indicate the efficiency of a precursor for the formation of several different metabolites.

tion reaction. The utilization of a labeled compound as a source of acetyl can therefore be determined by an isotope analysis of the acetyl derivative excreted in the urine. Bloch and Rittenberg (9) had shown that acetic acid itself acetylates *p*-aminobenzoic acid as well as  $\gamma$ -phenylaminobutyric acid, *i.e.* both aromatic and aliphatic foreign amines. They found on the other hand that alanine, presumably by way of pyruvic acid, is capable of providing the acetyl group for the acetylation of  $\gamma$ -phenylaminobutyric acid only, but not for *p*-aminobenzoic acid.

TABLE I

*Isotope Concentrations of Urinary Excretion Products after Feeding Labeled Pyruvic and Acetic Acids*

Experiment No.	Compound fed			Acetyl groups of acetyl-					
		Per 100 gm. per day	Isotope concentration*	<i>p</i> -Aminobenzoic acid			$\gamma$ -Phenylaminobutyric acid		
				RIC†	CC†	SCC†	RIC	CC	SCC
				per cent	per cent	per cent	per cent	per cent	per cent
1†	2-C <sup>14</sup> pyruvic acid	0.46	8,400	0.14	0.32	0.21	2.0	4.3	2.9
2†	1-C <sup>14</sup> acetic "	0.45	12,600	3.5	7.6	7.6	2.7	5.9	5.9
3§	2-C <sup>14</sup> pyruvic "	0.50	12,300	1.0	2.0	1.3	1.1	2.0	1.3
3§	1-C <sup>13</sup> acetic "	0.47	11.9				2.1	4.3	4.3
4§	2-C <sup>14</sup> pyruvic "	0.50	12,300	1.2	2.4	1.6	0.77	1.5	1.0
4§	1-C <sup>13</sup> acetic "	1.04	11.9	4.6	4.4	4.4	4.9	4.7	4.7
5§	1-C <sup>13</sup> pyruvic "	1.07	17.0	0.19	0.18	0.18			

\* Counts per minute of BaC<sup>14</sup>O<sub>3</sub>, or atom per cent excess C<sup>13</sup>.

† RIC = relative isotope concentration, CC = concentration coefficient, and SCC = specific concentration coefficient.

‡ Rats of the laboratory strain.

§ Rats of Sprague-Dawley strain.

Similar results were obtained after feeding labeled pyruvic acid to rats of the laboratory strain. The analytical values (Table I) indicate in particular that there is no significant conversion of pyruvic acid to acetic acid, as in this strain the isotope concentration of acetyl-*p*-aminobenzoic is very low. From the experiment with carboxyl-labeled pyruvic acid it is clear that only a negligible fraction of the carboxyl carbon atoms of pyruvic acid is incorporated into the acetyl groups. Only carbon atoms 2 and 3 of pyruvic acid are therefore utilized in the acetylation reaction. The use of the "specific concentration coefficients" for these data is thus justified, the factor *f* being equal to 0.67.

In the Sprague-Dawley strain, the acetyl groups of  $\gamma$ -phenylaminobutyric acid and *p*-aminobenzoic acid have nearly equal isotope concentrations, indicating a considerable difference of pyruvic acid metabolism in the

two strains. This result would seem to indicate that a portion of the fed pyruvic acid is decarboxylated to acetic acid, which in turn provides the acetyl groups for both foreign amines. The possibility cannot be excluded that Sprague-Dawley rats are capable of using pyruvic acid directly, *i.e.*, without conversion to acetic acid, for the acetylation of *p*-aminobenzoic acid. However, this explanation seems less plausible. If large quantities of labeled acetic acid are formed from the ingested pyruvic acid, it becomes impossible to evaluate the direct incorporation of carbon atoms of pyruvic acid into other compounds, such as fatty acids and cholesterol, for which acetic acid too is a precursor (3).

*Pyruvic Acid Pool*—A calculation of the hepatic pyruvic acid pool and its isotope concentration can be attempted from the data obtained in the laboratory strain, since after feeding pyruvic acid, the isotope content of the acetyl group of acetyl-*p*-aminobenzoic acid is very small, and therefore no appreciable conversion of pyruvic acid to acetic acid could have taken place. Therefore the appearance of the label in the acetyl group of acetyl- $\gamma$ -phenylaminobutyric acid will be the result of direct acetylation by pyruvic acid; *i.e.*, condensation of pyruvic acid with the amine and subsequent decarboxylation of the condensation product (18). The isotope concentration of this acetyl group depends on the isotope concentration of the pyruvic acid in the metabolic pool and on the relative proportion of acetyl groups of  $\gamma$ -phenylaminobutyric acid derived from pyruvic and acetic acids respectively.

It can be seen (Table I) that after feeding labeled acetic acid the relative isotope concentrations of the acetyl groups of acetyl-*p*-aminobenzoic acid and acetyl- $\gamma$ -phenylaminobutyric acid in the laboratory strain are not identical. The acetyl group of acetyl-*p*-aminobenzoic acid is derived from acetic acid only and thus provides a measure of the isotope concentration of the acetic acid pool, while the isotopic concentration of the acetyl group of acetyl- $\gamma$ -phenylaminobutyric acid is lower. If it is assumed that this difference is due to direct acetylation by pyruvic acid, then the relative contribution of acetic and pyruvic acids for the acetylation of phenylaminobutyric acid can be calculated.

$$R = \frac{I'_a}{\bar{I}_a - I'_a}$$

where  $R$  is the ratio of acetylation of  $\gamma$ -phenylaminobutyric acid by acetic to that by pyruvic acid,  $\bar{I}_a$  is the isotope concentration of the acetyl group of acetyl-*p*-aminobenzoic acid, and  $I'_a$  is the isotope concentration of the acetyl group of acetylphenylaminobutyric acid.

From the data of Experiment 2 (Table I)  $R$  is found equal to 3.5, *i.e.*, acetic acid acetylates  $\gamma$ -phenylaminobutyric 3.5 times as fast as does

pyruvic acid. From this ratio and from the isotope concentration of the acetyl group of acetyl- $\gamma$ -phenylaminobutyric acid after feeding labeled pyruvic acid, the isotope concentration of the pyruvic acid actually employed for acetylation and therefore the isotope concentration in the metabolic pool can be calculated.

$$I_p = I'_p(R + 1)$$

where  $I_p$  is the isotope concentration of the pyruvic acid pool, and  $I'_p$  is the isotope concentration of the acetyl group of phenylaminobutyric acid. The "relative isotope concentration" of the pyruvic acid in the pool is calculated to be 13 per cent.

The size of the pyruvic acid pool is then given by

$$P_p = \frac{(i_p - I_p)}{I_p} F_p$$

where  $P_p$  is the pyruvic acid pool in millimoles per 100 gm. of rat tissue per day,  $i_p$  is the isotope concentration of administered pyruvic acid, and  $F_p$  is the quantity of administered pyruvic acid in millimoles per day.

If this calculation is carried out, it is found that the pyruvic acid pool is about 6 to 7 mM per 100 gm. per day. Since this calculation depends on several isotope determinations in different animals, it can be considered indicative of an order of magnitude only.

In comparison the acetic acid pool is found to be about 12 to 15 mM per 100 gm. per day in the laboratory strain and about 20 to 25 mM per 100 gm. per day in the Sprague-Dawley strain. These values are in good agreement with the results obtained by Bloch and Rittenberg (9).

The size of the pyruvic acid pool is far smaller than the quantity of carbohydrate in the diet. It is comparable to the amount of pyruvic acid which could arise from a quantity of antiketogenic amino acid equivalent to that contained in the dietary protein. It would appear, therefore, that in the liver only a small part of the administered carbohydrate is in equilibrium with pyruvic acid in the laboratory strain.

For the Sprague-Dawley strain a similar calculation of the pyruvic acid pool cannot be made, since pyruvic acid provides labeled acetyl groups also for *p*-aminobenzoic acid. Therefore it is not possible to determine the relative contributions of pyruvic and acetic acids to the isotope content of the acetyl groups of  $\gamma$ -phenylaminobutyric acid.

*Liver Glycogen*—The incorporation of isotopic carbon from labeled pyruvic acid into liver glycogen is quite variable in different animals but lies within the same range in both rat strains. The half life time of liver glycogen was determined by Stetten and Boxer (2) to be about 1 day. On the basis of this value about 85 per cent of the liver glycogen should have

been replaced by newly formed glycogen in the 3 day feeding period. From the data in Table II it can be estimated that in the laboratory strain the isotope concentration of the liver glycogen is only about one-thirtieth the isotope concentration of the pyruvic acid pool. A major part of the liver glycogen must therefore have been derived from unlabeled sources, presumably from dietary carbohydrate directly. This observation is in agreement with the results obtained by Vennesland *et al.* (19), who observed that the extra glycogen deposited after administration of labeled lactic acid to fasted rats contained only a small fraction of the label.

The isotope from carbonyl- as well as from carboxyl-labeled pyruvic acid is incorporated into liver glycogen to an equal extent, indicating the utilization of all carbon atoms of pyruvic acid for glycogen formation.

TABLE II  
*Isotope Concentrations of Liver Glycogen and Urea after Feeding Labeled Pyruvic and Acetic Acids*

The results are expressed in per cent.

Experiment No.	Compound fed	Liver glycogen		Urea		
		RIC	CC	RIC	CC	SCC
1*	2-C <sup>14</sup> pyruvic acid	0.17	0.38			
2*	1-C <sup>14</sup> acetic "	0.03	0.06			
3†	2-C <sup>14</sup> pyruvic "	0.26	0.51	0.43	0.84	0.28
4†	2-C <sup>14</sup> " "	0.16	0.32	0.41	0.83	0.28
4†	1-C <sup>13</sup> acetic "	0.13	0.12	0.50	0.49	0.25
5†	1-C <sup>13</sup> pyruvic "	0.48	0.41	0.85	0.80	0.27

\* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

Acetic acid carbon is incorporated to a small extent only into the liver glycogen.

*Urea*—From experiments by Mackenzie and du Vigneaud (20) it is known that the urea carbon and the respiratory carbon dioxide have the same isotope concentrations. The "specific concentration coefficients" were calculated after the administration of 2-C<sup>14</sup> pyruvic acid, 1-C<sup>13</sup> pyruvic acid, and 1-C<sup>13</sup> acetic acid to rats of the Sprague-Dawley strain, with  $f = \frac{1}{3}$  for the pyruvic acids and  $f = \frac{1}{2}$  for acetic acid. These data are given in Table II and suggest an analogous rate of metabolism for pyruvic and acetic acids in these animals.

*Glycine*—Hippuric acid was isolated from the urine of rats which had been fed benzoic acid together with carbonyl- or carboxyl-labeled pyruvic acid or carboxyl-labeled acetic acid. The "relative isotope concentrations" given in Table III indicate that acetic acid is not a precursor for glycine.



The isotope concentration of labeled glycine was of the same order of magnitude after feeding either carbonyl- or carboxyl-labeled pyruvic acids. Degradation by ninhydrin (21) of the glycine obtained after feeding carbonyl-labeled pyruvic acid indicates that over 80 per cent of the isotope was present in carbon atom 2. It is therefore concluded that carbon atoms 1 and 2 of pyruvic acid are the precursors of carbon atoms 1 and 2 of glycine respectively. In view of the fact that Shemin (10) has demonstrated the conversion of serine to glycine, it seems most reasonable to assume that the utilization of carbon atoms 1 and 2 of pyruvic acid for glycine synthesis proceeds via serine as an intermediate. Chargaff and Sprinson (22) have proposed a mechanism for the conversion of serine to pyruvic acid. If this reaction series were reversible, it could account for the results obtained in this experiment. A similar observation was made by Buchanan *et al.* (23) who observed the conversion of lactic acid to

TABLE III  
*Incorporation of Isotopic Carbon into Glycine from Labeled Acetic and Pyruvic Acids*

Experiment No.	Compound fed			Glycine from hippuric acid	
		Per 100 gm. per day	Isotope concentration*	RIC	CC
		<i>msr</i>		<i>per cent</i>	<i>per cent</i>
5†	1-C <sup>14</sup> acetic acid	0.22	87,000	0.008	0.04
5†	1-C <sup>13</sup> pyruvic "	1.1	17.0	0.34	0.25
6†	2-C <sup>14</sup> " "	0.5	12,300	0.35	0.70

\* Counts per minute of BaC<sup>14</sup>O<sub>3</sub> or atom per cent excess C<sup>13</sup>.

† Rats of Sprague-Dawley strain.

glycine in the pigeon. Comparing the isotope concentrations of the different carbon atoms of uric acid after labeled glycine feeding with labeled lactic acid administration, the authors reached the conclusion that carbon atoms 1 and 2 of lactic acid are utilized for glycine formation in the pigeon.

*Cholesterol*—Experiments by Bloch and Rittenberg (4) have demonstrated the importance of acetic acid as a precursor of cholesterol. The contribution of acetic acid carbon atoms for the formation of liver cholesterol in the present experiments can be estimated from the turnover rate and the concentration of the acetic acid pool. It can be assumed that about 30 per cent of the liver cholesterol was newly synthesized in the 3 day feeding period, based on a half life time of 5 to 6 days (5, 9). The fraction derived from the carboxyl carbon atom of acetic acid is calculated to be 27 to 33 per cent of all carbon atoms of cholesterol in both rat strains. This value is in good agreement with the results obtained by Bloch and

Rittenberg (4) who found acetic acid to provide over 50 per cent of the carbon atoms of cholesterol. Experiments by these authors (5) indicate a larger utilization of the methyl than of the carboxyl carbon atom of acetic acid.

By a similar calculation it can be estimated that in the laboratory strain of rats about 7 to 8 per cent of the cholesterol carbon is derived from the carbonyl carbon atom of pyruvic acid. In this instance direct utilization of pyruvic acid is probable because, as has been shown, no conversion of pyruvic to acetic acid occurs in the laboratory strain. The extent of incorporation of pyruvic acid carbon atoms cannot be calculated in the Sprague-Dawley strain, as decarboxylation of pyruvic to acetic acid does occur for reasons mentioned above.

TABLE IV

*Isotope Concentrations of Liver and Carcass Cholesterol after Feeding Labeled Pyruvic and Acetic Acids*

The results are expressed in per cent.

Experiment No.	Compound fed	Liver cholesterol		Carcass cholesterol	
		RIC	CC	RIC	CC
1*	2-C <sup>14</sup> pyruvic acid	0.39	0.85	0.10	0.23
2*	1-C <sup>14</sup> acetic "	0.66	1.5	0.10	0.23
3†	2-C <sup>14</sup> pyruvic "	0.16	0.31	0.14	0.28
4†	2-C <sup>14</sup> " "	0.09	0.18	0.08	0.15
4†	1-C <sup>13</sup> acetic "	0.77	0.73	0.12	0.11
5†	1-C <sup>13</sup> pyruvic "	0.14	0.13		

\* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

From experiments in the Sprague-Dawley strain with carboxyl-labeled pyruvic acid, it appears probable that pyruvic acid as a whole is utilized to some extent for cholesterol synthesis. After feeding carboxyl-labeled pyruvic acid the "relative isotope concentration" of liver cholesterol was found to be about half of that which is obtained after administration of carbonyl-labeled pyruvic acid. The low analytical value does not permit a quantitative evaluation of this finding but suggests at least a partial utilization of the carboxyl carbon atom of pyruvic acid.

In view of the uncertainty of the contributions of the methyl carbon atom of acetic acid and of the methyl and carboxyl carbon atoms of pyruvic acid in these experiments, the "concentration coefficients" only are reported in Table IV. It is therefore not possible to set up a complete carbon balance from the data. Other precursors can be involved to a small extent only in the synthesis of cholesterol.

The "relative isotope concentrations" of the carcass cholesterols are found to be only slightly lower than those of the liver cholesterols. Since in adult animals the isotope concentration of liver cholesterol is found to be several times the isotope content of carcass cholesterol, the liver has been suggested as the site of cholesterol synthesis (9). The similarity of the values found in the experiments reported here is most probably due to the use of growing animals. In an unreported experiment carried out with an adult rat under comparable conditions, the isotope concentration of the carcass cholesterol is only one-third of the liver cholesterol.

*Fatty Acids*—Rittenberg and Bloch (5) have found that after feeding carboxyl-labeled acetic acid only the odd carbon atoms of the isolated

TABLE V

*Isotope Concentrations of Liver and Carcass Fatty Acids after Feeding Labeled Pyruvic and Acetic Acids*

The results are expressed in per cent.

Experiment No.	Compound fed	Liver fatty acids						Carcass fatty acids					
		Saturated			Unsaturated			Total			Saturated		
		RIC	CC	SCC	RIC	CC	SCC	RIC	CC	SCC	RIC	CC	SCC
1*	2-C <sup>14</sup> pyruvic acid	1.1	2.3	1.5	0.45	0.99	0.66	0.11	0.25	0.17	0.16	0.34	0.22
2*	1-C <sup>14</sup> acetic "	0.71	1.6	1.6	0.22	0.49	0.49	0.06	0.13	0.13	0.05	0.11	0.11
3†	2-C <sup>14</sup> pyruvic "	0.42	0.83	0.56	0.15	0.30	0.20	0.16	0.32	0.21	0.21	0.42	0.28
4†	2-C <sup>14</sup> " "	0.41	0.81	0.54	0.20	0.39	0.26	0.15	0.30	0.20	0.20	0.40	0.27
4†	1-C <sup>14</sup> acetic "	1.7	1.6	1.6	0.42	0.40	0.40	0.29	0.29	0.29	0.52	0.50	0.50
5†	1-C <sup>13</sup> pyruvic "	0.0											

\* Rats of the laboratory strain.

† Rats of Sprague-Dawley strain.

fatty acids contained the label. They concluded that their finding made it mandatory to assume that fatty acids are synthesized from C<sub>2</sub> units, that acetic acid is a precursor for C<sub>2</sub> units, and that the C<sub>2</sub> units from acetic acid are utilized at random for the entire fatty acid chain. This concept necessarily requires formation of C<sub>2</sub> units from all other fatty acid precursors and random utilization of these C<sub>2</sub> units. Rittenberg and Bloch considered that the C<sub>2</sub> units from acetic acid are not necessarily identical to the C<sub>2</sub> units from other precursors and suggested the formation of acylpyruvic acids as intermediates with subsequent decarboxylation of the condensation products.

The experiments presented here are in full agreement with the hypothesis of Rittenberg and Bloch. The data are given in Table V. After feeding carboxyl-labeled pyruvic acid the isotope concentration of the liver fatty

acids was found to be negligible. From this result it can be concluded that only carbon atoms 2 and 3 of pyruvic acid are used in fatty acid synthesis. Decarboxylations of saturated fatty acids isolated after feeding carbonyl-labeled pyruvic acid yielded carbon dioxide, the isotope concentration of which was about twice that of the fatty acids. This indicates that the carbonyl carbon atom of pyruvic acid is incorporated in a random manner into the odd numbered positions of the fatty acid chain (Table VI). Accordingly, in Table V the "specific concentration coefficients" are calculated with  $f = 0.67$ .

TABLE VI

*Distribution of Isotopic Carbon in Fatty Acid Chains after Administration of Labeled Pyruvic Acid*

Experiment No.	Isotope concentration		
		Found	Calculated (b)
		<i>counts per min.</i>	<i>counts per min.</i>
7*	Saturated fatty acids, carcass	121(a)	
	Carbon dioxide, decarboxylation	225	241†
	Ketone, decarboxylation	119	118‡
1	Saturated fatty acids, liver	87(a)	
	Carbon dioxide, decarboxylation	156	174†
	Ketone, decarboxylation	90	84‡

\* In this experiment pyruvic acid was injected.

† Calculated from  $b = 2a$ .

‡ Calculated from  $b = (32/33)a$ , assuming the average chain length of the fatty acids to be 17 carbon atoms.

In the laboratory strain of rats, in which it is possible to calculate the isotope concentration of the pyruvic and acetic acid pools, an estimate of the extent of each compound as a precursor for the fatty acids of liver can be made. With a value of 1.9 days for the half life time of the liver fatty acids (4), about two-thirds of the maximum isotope concentration will have been reached in the 3 day feeding period. On this basis and from the isotope concentrations of the metabolic pools it is estimated that about one-sixth of the carbon atoms of the saturated liver fatty acids has been derived from pyruvic acid and about one-third from acetic acid; i.e., the ratio of incorporation of pyruvic to acetic acid carbon atoms is about 1:2. This ratio which is independent of the half life time may probably be significant for the mechanism of fatty acid synthesis. In the present experiments acetic and pyruvic acids account for only about one-half of the carbon atoms of the saturated liver fatty acids. The remaining carbon atoms may have originated from such unlabeled sources as the carcass

fatty acids or the dietary cod liver oil. On a completely fat-free diet a larger portion of the saturated liver fatty acids could possibly arise by synthesis.

The isotope concentrations of the unsaturated liver fatty acid have little significance in themselves because this fraction contains also the multiply unsaturated acids. The latter are essential; they are not synthesized in the animal (24) and therefore do not contain the label. The isotope concentration of the oleic acids will therefore be considerably higher than the total unsaturated fraction and may even approach the tracer concentration of the saturated fatty acids in the liver. However, the ratio of incorporation of pyruvic to acetic acid carbon into the unsaturated fatty acids can be determined. In the laboratory strain this ratio is about 4:5. It differs sufficiently from the ratio for the saturated acids to suggest a possible difference of the respective synthetic mechanisms (25).

A similar estimation of the incorporation of pyruvic acid carbon into the liver fatty acids of the Sprague-Dawley rats cannot be carried out in view of the conversion of pyruvic acid to acetic acid in these animals. However, the comparison of isotope ratios gives some indirect information. In Experiment 4 (Table IV) in which pyruvic and acetic acids had been fed to the same rat, the ratio for liver cholesterol is

$$\frac{CC \text{ (pyruvic acid)}}{CC \text{ (acetic acid)}} = 0.25$$

for the saturated liver fatty acids (Table V) it is

$$\frac{CC \text{ (pyruvic acid)}}{CC \text{ (acetic acid)}} = 0.5$$

and for the unsaturated liver fatty acids (Table V) it becomes

$$\frac{CC \text{ (pyruvic acid)}}{(CC \text{ acetic acid)}} = 1$$

The higher ratios found in the liver fatty acids are most readily interpreted by assuming direct incorporation of pyruvic acid carbon atoms. If the labeled carbon of pyruvic acid were to enter the isolated compounds only after conversion of pyruvic acid into acetic acid, an identical ratio in all metabolites would have to be expected.

In both strains, the total and the saturated carcass fatty acids are found to have approximately identical isotope concentrations. As the carcass fat contains only a small quantity of multiply unsaturated fatty acids (26), the oleic acid fraction will have about the same isotope concentration as the saturated fatty acids. These results are in contrast to the experiments carried out with heavy water (27) in which the deuterium concentration of the unsaturated fraction is considerably lower than that of the saturated fatty acids. This difference is not due to the use of growing

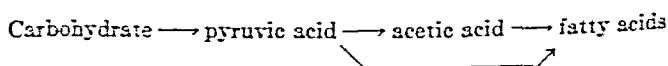
animals, because in the experiment with an adult rat under identical conditions the total and saturated fatty acids also have identical isotope contents. It seems most plausible to attribute the variations of experiments with labeled hydrogen and labeled carbon to different mechanisms of synthesis of saturated and unsaturated fatty acids (25). Such differences of the mechanisms were indicated by the unequal ratios of incorporation of acetic and pyruvic acid carbon atoms into saturated and unsaturated liver fatty acids and might easily account for the unequal uptake of deuterium from the body water.

#### DISCUSSION

Considerable differences were observed after feeding labeled pyruvic acid to two strains of rats, the laboratory strain and the Sprague-Dawley strain. In contrast the acetic acid metabolism proved to be nearly identical in both. The differences observed in the two strains can be most readily accounted for, if the assumption is made that the Sprague-Dawley rats convert pyruvic acid to acetic acid on a high carbohydrate diet, whereas the laboratory rats do not do so. In this connection the observation by Krah1 and Cori (28) may be relevant. These authors found that Sprague-Dawley rats are much more resistant to the production of alloxan diabetes, as measured by the increase of the blood glucose level, than another strain. It may perhaps be speculated that this observation can be attributed to two competing pathways of pyruvic acid metabolism; namely, conversion to acetic acid and to glucose respectively.

Experiments with heavy water by Schoenheimer *et al.* had shown that on a high carbohydrate diet the depot fat turns over at a rate requiring the daily synthesis of considerable quantities of fatty acids from small molecules. From similar heavy water experiments Stetten and Boxer (2) concluded that 35 per cent of dietary glucose is metabolized by way of fatty acids. However, the experiments with isotopic carbon reported here lead to the conclusion that the conversion of carbohydrate to fat by way of pyruvic acid accounts for a small fraction only of the metabolism of dietary carbohydrate. The fate of the major part of carbohydrate metabolism remains unaccounted for.

In the laboratory strain, pyruvic acid accounts for only about one-third of the carbon atoms of the saturated and for about four-ninths of the unsaturated liver fatty acids synthesized. The other carbon atoms are provided by acetic acid. In the Sprague-Dawley strain the metabolic sequence



may account for a somewhat larger part of carbohydrate metabolism.

The author is indebted to Mrs. Ruth Raper for valuable assistance in the course of this work.

## SUMMARY

Carbonyl- and carboxyl-labeled pyruvic acids and carboxyl-labeled acetic acid were synthesized and fed to two strains of rats.

Acetyl derivatives of *p*-aminobenzoic and  $\gamma$ -phenylaminobutyric acids were extracted from the urine; glycogen, cholesterol, and fatty acid were isolated from the liver and cholesterol and fatty acid from the carcass.

In the Sprague-Dawley strain conversion of pyruvic acid to acetic acid seems to occur.

In the laboratory strain no significant conversion of pyruvic acid to acetic acid was found. An estimate of the pyruvic acid pool could be made, indicating that pyruvic acid is not in complete equilibrium with dietary carbohydrate. Pyruvic and acetic acid carbon atoms are incorporated into fatty acids. On a quantitative basis acetic acid provides a larger number of carbon atoms for the fatty acids than pyruvic acid.

The utilization of pyruvic acid as a precursor for cholesterol is small.

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# ON THE METABOLIC FATE OF PYRUVAMIDE AND ACETAMIDE\*

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(Received for publication, July 12, 1948)

The method used for the synthesis of labeled pyruvic acid as reported in the preceding communication (1) involved the isolation of labeled pyruvamide as an intermediate and suggested an investigation of the fate of this compound in relation to pyruvic acid. Pyruvamide showed a marked difference from pyruvic acid in its metabolic behavior. In addition, an experiment with labeled acetamide was carried out for comparison.

## EXPERIMENTAL

The synthesis of pyruvamide and the isolation of body constituents were carried out as previously described (2).

*Acetamide*—Carboxyl-labeled potassium acetate was converted to acetyl bromide (1) and this compound added to an excess of liquid ammonia. After evaporation of the excess ammonia, the residue was extracted with hot ethyl acetate from which acetamide crystallized out after cooling. Yield, about 50 per cent; m. p. 78–79°.

*Formic Acid*—The urine was acidified with sulfuric acid, extracted with ether for 12 hours, and the ether extract evaporated to dryness. The residue was taken up in water, acidified, and treated with dinitrophenylhydrazine. The hydrazones were extracted with ethyl acetate and discarded. The remaining water layer was distilled with steam. The distillate was neutralized with sodium hydroxide, concentrated to a volume of 5 ml., and filtered. The *p*-bromophenacyl ester was then prepared as described by Hurd and Christ (3).

## Results

*Pyruvamide Feeding*—Data showing the incorporation of isotopic carbon into a number of metabolites after pyruvic acid, pyruvamide, and acetic acid feedings to rats of a laboratory strain are presented in Table I. The significance of the isotope concentrations obtained in the various isolated compounds after administration of pyruvic and acetic acids has been discussed previously (2). In particular it was found that in the laboratory strain only a negligible amount of pyruvic acid is converted to acetic acid.

\* This work was supported by a grant from the United States Public Health Service.

It can be seen by inspection of Table I that in all isolated compounds the distribution of the labeled carbon from pyruvamide is practically identical to that found after the administration of acetic acid. The results, however, differ significantly from those obtained after feeding pyruvic acid. Pyruvamide in contrast to pyruvic acid is a source of acetyl groups for acetyl-*p*-aminobenzoic acid exactly as is acetic acid. Pyruvic acid contributes less labeled acetyl for the acetylation of  $\gamma$ -phenylaminobutyric acid than does either acetic acid or pyruvamide. The isotope contents of glycogen and cholesterol, isolated from the liver, are similar after pyruvamide and acetic acid feeding but differ distinctly from that obtained after administration of pyruvic acid. In experiments in which pyruvic acid

TABLE I

*Isotope Concentrations of Body Constituents after Feeding Labeled Pyruvic Acid, Pyruvamide, and Acetic Acid*

Compounds isolated	Relative isotope concentrations* after feeding†		
	Pyruvic acid‡	Pyruvamide‡	Acetic acid
	per cent	per cent	per cent
Acetyl group of acetyl- <i>p</i> -aminobenzoic acid . . .	0.10	2.1	3.5
“ “ “ acetylphenylaminobutyric acid. . .	1.3	2.6	2.7
Liver glycogen . . . . .	0.12	0.05	0.03
“ cholesterol . . . . .	0.26	0.54	0.66
“ saturated fatty acids . . . . .	0.73	0.66	0.71

\*  $RIC = \frac{\text{specific radioactivity of isolated compound}}{\text{specific radioactivity of fed compound}}$

† 0.46 mm per day for 3 days.

‡ Radioactivity of pyruvamide and pyruvic acid calculated as present in 2 carbon atoms only.

and pyruvamide were injected rather than fed, a similar pattern of distribution of the label was obtained. These results may be taken as evidence that pyruvamide undergoes splitting between carbon atoms 1 and 2 in the body and that this splitting reaction occurs at a considerably faster rate than hydrolysis to pyruvic acid. The possibility was tested that the splitting of pyruvamide yields, besides acetic acid, a fragment consisting of the carboxyl carbon and amide nitrogen, which could contribute to the synthesis of urea. Carboxyl-labeled pyruvamide was therefore fed. The results shown in Table II indicate that the isotope concentration of urea did not differ from that of the expired carbon dioxide. It is therefore unlikely that the carboxyl carbon of pyruvamide is a specific precursor of urea. The analytical values from this experiment were considerably

below the isotope concentration of urea isolated after the administration of carboxyl-labeled pyruvic acid. This indicates that carbon atom 1 of pyruvamide is not readily converted to  $\text{CO}_2$ . From the urine of Sprague-Dawley rats fed normal pyruvamide, formic acid could be isolated and identified in the form of its *p*-bromophenacyl ester. Sonne *et al.* (4) have shown that formic acid is not converted to carbon dioxide in the pigeon. The occurrence of formic acid in the urine after pyruvamide feeding together with the low isotope concentration of carbon dioxide after feeding carboxyl-labeled pyruvamide suggests that the splitting of pyruvamide in the body yields acetic and formic acids as products.

*Pyruvamide in Liver Slices*—The utilization of carbon atoms of pyruvamide for the synthesis of fatty acids and cholesterol *in vitro* was determined under conditions which permit the demonstration of fatty acid synthesis in liver slices.<sup>1</sup> The data are given in Table III.

TABLE II

*Isotope Concentration of Urea and Respiratory Carbon Dioxide after Feeding Carboxyl-Labeled Pyruvamide*

	Isotope concentrations after feeding carboxyl- labeled pyruvamide*
	atom per cent excess $\text{C}^{13}$
$\text{CO}_2$ after 12 hrs.....	0.03
" " 30 " .....	0.03
" " 52 " .....	0.00
Urea, 1st 30 hrs. ....	0.03
" 2nd 22 " .....	0.00

\* 51 per cent excess  $\text{C}^{13}$  in the carboxyl carbon.

Bloch has shown that in this system the synthesis of cholesterol from labeled acetic acid in the presence of insulin is not materially changed by the addition of pyruvic acid. It can be seen from Table III that in the presence of pyruvic acid the isotope is incorporated to a similar extent from labeled pyruvamide or labeled acetic acid into the isolated cholesterol. If both labeled acetic acid and unlabeled pyruvamide are present, the isotope concentration of the cholesterol is lower. If it is assumed that acetic acid and pyruvamide can be utilized interchangeably, as can be concluded from the fact that individually both are utilized to a similar extent for cholesterol synthesis, the lower isotope value will be due to dilution of the labeled acetic acid by carbon atoms derived from the unlabeled pyruvamide. The experimental data are consistent with this assumption.

<sup>1</sup> Bloch, K., private communication.

Bloch and Kramer (5) have also shown that in the presence of insulin the incorporation of isotope into the fatty acids from labeled acetic acid is considerably enhanced if unlabeled pyruvic acid is added to the medium. The data in Table III indicate that pyruvamide can, at least partially, replace acetic acid as a source of carbon atoms for the synthesis of fatty

TABLE III

*Isotope Concentrations of Fatty Acids and Cholesterol after Addition of Labeled Pyruvamide and Acetic Acid to Rat Liver Slices*

1.5 gm. of liver slices in 16 ml. of Krebs-Ringer bicarbonate buffer at pH 7.4, containing 0.8 unit of insulin per ml. Incubated for 3 hours at 37° in O<sub>2</sub>-CO<sub>2</sub>.

Compounds isolated	Isotope concentrations* after addition of		
	0.12 mM 2-C <sup>14</sup> pyruvamide† and 0.18 mM pyruvic acid	0.12 mM 1-C <sup>14</sup> acetic acid and 0.18 mM pyruvic acid	0.12 mM 1-C <sup>14</sup> acetic acid and 0.18 mM pyruvamide
	per cent	per cent	per cent
Fatty acids.....	0.07	0.18	0.008
Cholesterol.....	0.8 ± 0.3	0.7 ± 0.1	0.24

\* Per cent specific radioactivity of added compounds.

† Radioactivity of pyruvamide calculated as present in 2 carbon atoms only.

TABLE IV

*Isotope Concentrations after Administration of Labeled Acetic Acid and Acetamide*

Compounds isolated	Relative isotope concentrations* after feeding†	
	Acetic acid	Acetamide
	per cent	per cent
Acetyl group of acetyl- <i>p</i> -aminobenzoic acid..	4.4	2.0
Liver cholesterol .....	0.73	0.07
" saturated fatty acids .....	1.6	0.16
Urea ... ..	0.49	0.15

\*  $RIC = \frac{\text{specific radioactivity of isolated compound}}{\text{specific radioactivity of fed compound}}$ .

† 0.46 mM per day for 3 days.

acids. If in the experiments with labeled acetic acid normal pyruvamide is substituted for pyruvic acid, only a negligible uptake of labeled carbon into the fatty acids is observed.

These results are in accord with those obtained in the feeding experiments in showing that pyruvamide *in vitro* acts similarly to acetic acid but differs considerably from pyruvic acid. In this series of experiments too, pyruvamide seems to be split much faster than it is hydrolyzed.

*Acetamide Feeding*—The isotope concentrations obtained after feeding labeled acetamide and labeled acetic acid to Sprague-Dawley rats are given in Table IV. It can be seen that acetamide is only about one-tenth as effective as acetic acid as a precursor for fatty acids and cholesterol. This finding indicates that only a small fraction of acetamide carbon enters the acetic acid pool. The hydrolysis to acetic acid is probably a slow process.

On the other hand, however, acetamide is quite efficiently used for the acetylation of *p*-aminobenzoic acid, a value of about one-half of the isotope concentration of acetic acid being obtained. As the low value of isotope incorporation into fatty acids and cholesterol excludes the fact that acetic acid is formed in large amounts from acetamide, the assumption may be made that acetamide is capable of acetylating *p*-aminobenzoic acid directly, *i.e.* without prior hydrolysis.

#### SUMMARY

Evidence has been presented to indicate that in the intact animal pyruvamide is split into acetic acid and formic acid, rather than hydrolyzed to pyruvic acid. The splitting reaction must occur at a fast rate. In liver slices pyruvamide can substitute for acetic acid as precursor for cholesterol and fatty acids. The significance of pyruvamide as a normal metabolite is unknown.

Acetamide is only partially and slowly hydrolyzed to acetic acid. The possibility exists that it may acetylate *p*-aminobenzoic acid without being hydrolyzed.

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# NAPHTHOQUINONE ANTIMALARIALS

## XXI. ANTISUCCINATE OXIDASE ACTIVITY\*

By HANS HEYMANN† AND LOUIS F. FIESER

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(Received for publication, May 10, 1948)

Ball, Anfinsen, and Cooper<sup>1</sup> studied a few representative 2-hydroxy-3-alkyl-1,4-naphthoquinones under investigation as antimalarial drugs with results that indicated a probable parallelism between *in vivo* activity in the inhibition of respiration of succinate oxidase and antimalarial activity as measured by assays in ducks. With kind guidance from Dr. Ball, we installed his test procedure and have investigated the possible parallelism in further detail.

### EXPERIMENTAL

The inhibitory effect of naphthoquinones on mixtures of cytochrome *c* and dehydrated succinate oxidase from beef heart in a phosphate buffer containing sodium succinate was studied manometrically according to Ball's technique.<sup>1</sup> The compounds studied will be indicated by their code numbers; the structures are given in Table I. A typical dehydrated enzyme preparation at a concentration of 900 mg. per liter respired at the rate of 107 c.mm. per 30 minutes when freshly prepared, and at the rate of 86 c.mm. per 30 minutes 1 month later; as the aging progressed, more and more drug was required to produce 50 per cent inhibition of the oxygen consumption (*e.g.*, 0.54 mg. of M-1916 per liter instead of the original 0.46 mg. per liter). This effect seems attributable to the antagonism to drug action exerted by the increased amounts of enzymatically inert protein in the aged preparations. Thus portions of an old, completely inactive enzyme preparation added to the test solutions of a fresh preparation and standard drug (M-1916) caused a progressive increase in the drug required

\* This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University, and in part with the aid of a grant from the Rockefeller Foundation.

With the technical assistance of Louise Wiarda and Jean B. Knowlton.

Papers I to XVII, *J. Am. Chem. Soc.*, **70**, 3151-3244 (1948); Papers XVIII to XX, *J. Pharmacol. and Exp. Therap.*, **94**, 85-124 (1948).

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<sup>1</sup> Ball, E. G., Anfinsen, C. B., and Cooper, O., *J. Biol. Chem.*, **168**, 257 (1947).

to half inhibit the system, and added plasma protein exerts a similar antagonism. However, neither the variation in a given enzyme preparation with time nor the variation from preparation to preparation was found

TABLE I

*Relative Antirespiratory Activity in Inhibition of Succinate Oxidase (37°)*

Standard, M-1916 = 1.

Code No.	2-Hydroxy-1,4-naphthoquinone, side chain	Relative potency, weight basis	Activity against <i>P. lophurae</i> , ED <sub>50</sub>
M-2261	—(CH <sub>2</sub> ) <sub>3</sub> -α-Acenaphthyl	9.7	
M-295	—(CH <sub>2</sub> ) <sub>3</sub> -β-Tetralyl	6.6	39.3
M-2254	—(CH <sub>2</sub> ) <sub>3</sub> -α-Naphthyl	5.3	>20
-2255	—(CH <sub>2</sub> ) <sub>3</sub> -9-(1,2,3,4-Tetrahydrophenanthryl)	5.1	>80
-2243	—CH <sub>2</sub> CH(CH <sub>3</sub> )CH <sub>2</sub> -Cyclohexyl	4.4	17.4
M-297	—(CH <sub>2</sub> ) <sub>3</sub> -β-Decalyl	4.3	8.6
M-333	—CH <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	4.0	7.7
M-1971	—(CH <sub>2</sub> ) <sub>4</sub> -Cyclohexyl	3.8	11.4
M-289	—(CH <sub>2</sub> ) <sub>3</sub> CH=CH <sub>2</sub>	3.8	14.3
M-2257	—(CH <sub>2</sub> ) <sub>3</sub> -5-Hydrindyl	3.1	34.7
M-273	—C <sub>10</sub> H <sub>21</sub> -n	2.6	20.4
M-285	—CH <sub>2</sub> CH(CH <sub>3</sub> )—C <sub>6</sub> H <sub>13</sub> -n	2.5	4.4
M-287	—(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	2.2	16.4
M-374	—(CH <sub>2</sub> ) <sub>3</sub> -Δ <sup>2</sup> -Cyclohexenyl	1.7	23.6
M-1714	—C <sub>15</sub> H <sub>31</sub> -n	1.2	43
M-1936	—(CH <sub>2</sub> ) <sub>12</sub> -Cyclopentyl	1.2	87
M-2246	—(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> )-Cyclohexyl	1.1	39
M-1916	—(CH <sub>2</sub> ) <sub>3</sub> -Cyclohexyl	1.0	21.4
M-2237	—(CH <sub>2</sub> ) <sub>3</sub> -C <sub>6</sub> H <sub>4</sub> (CH <sub>3</sub> ) <sub>2</sub> -2,5	0.73	>75
M-1933	—(CH <sub>2</sub> ) <sub>2</sub> CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0.59	13.9
M-1929	—(CH <sub>2</sub> ) <sub>4</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0.56	16.1
M-1944	—CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0.35	5.6
M-2262	—CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>2</sub> -Cyclohexyl	0.13	
M-1955	—(CH <sub>2</sub> ) <sub>3</sub> C <sub>6</sub> H <sub>5</sub>	0.11	65
M-1711	—(CH <sub>2</sub> ) <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0.07	75
M-2263	—CH <sub>2</sub> -Menthyl (Isomer B)	0.07	
M-1963	“ ( “ A)	0.05	>100
M-2264	—Phytyl	0.04	
M-1523	—CH <sub>2</sub> CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0.03	68
M-1935	—C <sub>6</sub> H <sub>4</sub> Br-p	0.02	50

to interfere with the determination of the potencies of a series of naphthoquinones relative to that of the standard, M-1916. When the inhibitory effect of a given compound was studied, a parallel determination was always made of the effect of M-1916 on the same enzyme preparation, and a blank was run with each set of concentrations to offset variation in the concentra-



tion or activity of the different enzyme suspensions. The naphthoquinones give typical dosage-response curves that are linear up to about 70 per cent inhibition and then level off asymptotically. The drug activity is expressed as the concentration (by weight) necessary to cause 50 per cent inhibition of respiration ( $LD_{50}$ ). The relative potency is given by the ratio of the  $LD_{50}$  values found for M-1916 and the substance studied in parallel determinations. In a series of comparisons in which the  $LD_{50}$  for M-1916 varied from 0.46 to 1.07 mg. per liter, the following values were found for the relative potency of M-297: 4.1, 4.5, 4.5, and 5.3.

### Results

The activities found for twenty-nine naphthoquinones are listed in Table I in the order of decreasing relative potency. The *in vivo* activities against *Plasmodium lophurae* in ducks, as determined by A. P. Richardson, are reported in terms of the effective dose ( $ED_{95}$ ) (mg. per kilo) required to produce a 95 per cent reduction in parasitemia (see Paper II). Some correlations between inhibitory power and antimalarial activity (last column) can be discerned in a sharply defined series of compounds; for example, among the compounds with normal and isoalkyl side chains the activity increases with increasing carbon content from  $C_5$  to  $C_8$  (0.03, 0.07, 0.56, 2.2) and decreases from a  $C_{10}$  chain (2.6) to a  $C_{15}$  chain (1.2). The peak of antisuccinate oxidase activity seems to be about the same as that for antimalarial activity, but beyond the peak the loss in activity with increasing molecular weight is less abrupt. The four compounds of highest anti-respiratory activity all have side chains of high carbon content ( $C_{13}$  to  $C_{17}$ ).

However, when the results are considered as a whole, certain glaring discrepancies are observed between the *in vitro* and *in vivo* activities. M-295 is the second most active inhibitor and is 6.6 times as potent as M-1916, but it is only half as active as an antimalarial; M-1944 is only one-third as effective an inhibitor as M-1916 but is nearly 4 times as active as this substance in the duck assays. As a further test of the validity of experimentation with this enzyme system, examination was made of several compounds differing significantly in structure from the hydroxyalkylnaphthoquinones that show characteristic antiplasmodial activity. It was found that a hydroxyl or carboxyl group in the side chain results in a practically complete loss in antirespiratory activity, as well as in antiplasmodial activity, and that the introduction of a methyl group into the benzenoid nucleus of an active hydroxyalkylnaphthoquinone destroys the antisuccinate oxidase activity, whereas methyl substitution in the side chain has the opposite influence. In these subtle effects activity in the inhibition of the succinate oxidase parallels antimalarial activity. However, M-1923, the

chloride of hydrolapachol (M-1523) was found to have an antirespiratory potency 4.5 times as great as hydrolapachol, and yet assays in ducks showed the compound to be completely inactive. Another probable discrepancy is that  $\alpha$ -naphthoquinone showed marked activity as a respiration inhibitor, namely 46 per cent the potency of M-1916. It seems necessary to conclude that antirespiratory activity against the succinate oxidase system cannot be relied upon to give even an approximate index of antimalarial potency.

#### SUMMARY

Activity in the inhibition of the succinate oxidase system does not appear to provide a reliable guide to antimalarial activity *in vivo*.

# NAPHTHOQUINONE ANTIMALARIALS

## XXII. RELATIVE ANTIRESPIRATORY ACTIVITIES (PLASMODIUM LOPHURAE)\*

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(Received for publication, May 20, 1948)

In Papers XIX and XX of this series we have reported application of the procedure of Wendel<sup>1</sup> for determination of the activities of 2-hydroxy-3-alkyl-1,4-naphthoquinones in the inhibition of respiration of parasitized red blood cells to the study of naphthoquinone-protein interactions and to the investigation of metabolic drug deactivation. The present paper reports the results of the determination of the biological activities of an unusually extensive series of related compounds made available by a war time research.

### Results

The methodology was that described in Paper XIX. Table I records the antirespiratory activities of several series of compounds relative to that of compound M-1916. The relative potency is reported on a molar basis as calculated from the ratio,  $IC_{50}^m$  (M-1916)/ $IC_{50}^m$  (quinone studied), where  $IC_{50}^m$  is the molar concentration required to reduce respiration by 50 per cent. When more than one determination was made, the average deviation is recorded and the number of determinations is given in parentheses. In some instances the values reported are averages of fourteen to twenty-three comparisons that were conducted over a period of several months in the course of studies of protein antagonism and drug metabolism, and the average deviation is in the order of 15 to 25 per cent. For comparison with the molar *in vitro* activities in the inhibition of respiration of parasitized red blood cells, figures are given in the last column of Table I for the molar *in vivo* activities against *Plasmodium lophurae* in the duck relative to

\* This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University, and in part with the aid of a grant from the Rockefeller Foundation.

Papers I to XVII, *J. Am. Chem. Soc.*, **70**, 3151-3244 (1948); Papers XVIII to XX, *J. Pharmacol. and Exp. Therap.*, **94**, 85-124 (1948).

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<sup>1</sup> Wendel, W. B., *Federation Proc.*, **5**, 406 (1946).

TABLE I

*Relative Activity in Inhibition of Respiration of Parasitized Red Blood Cells at 41°*  
*(Plasmodium lophurae in Ducks)*

Standard, M-1916 = 1.

Code No.	Side chain, type	n	Relative molar activity	
			Antirespiratory*	In vivo
M-1709	—(CH <sub>2</sub> ) <sub>n</sub> CH <sub>3</sub>	3	0.05 ± 0.01 (3)	0.1
M-1710		4	0.15 ± 0.02 (2)	0.15
M-268		5	0.5 ± 0.02 (2)	0.2
M-260		6	0.5 ± 0.09 (3)	0.4
M-271		7	1.3 ± 0.1 (3)	0.6
-2275		8	2.1 ± 0.1 (3)	2.5
273		9	1.65 ± 0.2 (4)	1.1
M-1926		10	3.1 ± 0.1 (5)	0.6
M-1928		11	2.5 ± 0.1 (3)	0.9
M-1924		12	4.6 ± 0.2 (2)	0.5
M-2347		13	1.9 ± 0.1 (3)	Feeble
M-1714		14	0.51 ± 0.05 (2)	0.6
M-1706	—(CH <sub>2</sub> ) <sub>n</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	1	0.1	0
M-1523		2	0.3	0.3
M-1711		3	0.4	0.25
M-1929		4	1.4	1.2
M-287		5	1.7	1.25
M-2284		6	1.5	2.5
M-300		7	4.6	2.3
M-2287		8	3.2	1.8
M-1920	—(CH <sub>2</sub> ) <sub>n</sub> -Cyclopentyl	1	0.5	0.2
M-2321		2	0.7	0.6
M-2322		3	1.3	1.2
M-2331		4	1.1	1.3
M-2335		5	1.6	1.8
M-1914	—(CH <sub>2</sub> ) <sub>n</sub> -Cyclohexyl	1	1.4	0.35
M-1915		2	1.1	0.9
M-1916		3	1.00	1.00
M-1971		4	1.6 ± 0.1 (2)	2.0
M-1956		5	2.3	0.8
M-384	—(CH <sub>2</sub> ) <sub>n</sub> -4'-Cyclohexylcyclohexyl- <i>cis</i>	1	4.6 ± 0.2 (2)	2.75
M-2329		2	8.7 ± 0.2 (2)	0.6
M-2291		3	7.1 ± 0.3 (2)	1.9
M-380	—(CH <sub>2</sub> ) <sub>n</sub> -4'-Cyclohexylcyclohexyl- <i>trans</i>	1	4.2	3.8
M-2330		2	4.6	3.7
M-2292		3	6.5	3.8

TABLE I—Continued

Code No.	Side chain, type	n	Relative molar activity	
			Antirespiratory*	In vivo
M-297	—(CH <sub>2</sub> ) <sub>2</sub> -β-Decalyl- <i>trans</i>		1.6 ± 0.3 (4)	2.9
M-2279	—(CH <sub>2</sub> ) <sub>2</sub> -β-Decalyl- <i>cis</i>		4.1 ± 0.6 (5)	7.2
M-266	—Cyclohexyl		2.5 ± 0.36 (14)	2.3
M-2328	—β-Decalyl- <i>cis</i>		12.6 ± 2.0 (4)	4.4
M-2374	—β-Decalyl- <i>trans</i>		4.0 ± 0.2 (3)	1.8
M-2293	—4'-Cyclohexylcyclohexyl- <i>trans</i>		4.8 ± 1.0 (9)	36.2
M-1955	—(CH <sub>2</sub> ) <sub>n</sub> C <sub>6</sub> H <sub>5</sub>	3	0.3	0.3
M-2286		4	0.2	0.6
M-2276		5	0.4 ± 0.04 (2)	0.7
M-2387		6	0.8 ± 0.04 (2)	
M-2386		7	1.6 ± 0.1 (3)	
M-2382		8	1.7 ± 0.2 (3)	
M-2301		9	3.6 ± 0.4 (8)	1.95
M-1738	—(CH <sub>2</sub> ) <sub>n</sub> C <sub>6</sub> H <sub>4</sub> Cl- <i>p</i>	1	0	0.2
M-2289		2	0.7 ± 0.11 (14)	1.2
M-2260		3	0.8 ± 0.1 (2)	1.2
M-2340		5	2.5	0.9
M-2344		9	7.7 ± 0.2 (3)	3.1
M-2358	—(CH <sub>2</sub> ) <sub>n</sub> C <sub>6</sub> H <sub>4</sub> Br- <i>p</i>	2	0.4	0.9
M-2341		5	2.9	<0.6
M-2362		9	7.4	2.5
M-2346	—(CH <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>4</sub> CF <sub>3</sub> - <i>m</i>		0.1	0.2
M-2365	—(CH <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>10</sub> CF <sub>3</sub> -3'		0.91 ± 0.2 (3)	0.7
M-2380	—(CH <sub>2</sub> ) <sub>n</sub> C <sub>6</sub> H <sub>4</sub> OC <sub>6</sub> H <sub>5</sub> - <i>p</i>	1	0.14 ± 0.07 (5)	
M-2338		2	2.8 ± 0.1 (6)	0.85
M-2309		3	4.6 ± 0.8 (23)	1.75
M-2361		4	0.9 ± 0.07 (3)	Feeble
M-2345		5	2.5 ± 0.2 (4)	"
M-2360		9	2.7	1.0
M-2331	—(CH <sub>2</sub> ) <sub>8</sub> C(OH)(CH <sub>3</sub> ) <sub>2</sub>		0.2 ± 0.01 (2)	Weak
M-2343	—(CH <sub>2</sub> ) <sub>8</sub> C(OH)(C <sub>4</sub> H <sub>9</sub> - <i>n</i> ) <sub>2</sub>		2.1 ± 0.5 (3)	2.0†
M-2376	—(CH <sub>2</sub> ) <sub>6</sub> C(OH)(C <sub>6</sub> H <sub>13</sub> - <i>n</i> ) <sub>2</sub>		0.1 ± 0.02 (2)	<1.6†
M-2350	—(CH <sub>2</sub> ) <sub>8</sub> C(OH)(C <sub>5</sub> H <sub>11</sub> - <i>n</i> ) <sub>2</sub>		3.4 ± 0.8 (16)	7.8†
M-2367	—(CH <sub>2</sub> ) <sub>8</sub> C(OH)(C <sub>6</sub> H <sub>13</sub> - <i>n</i> ) <sub>2</sub>		1.3 ± 0.1 (2)	5.5†
M-2363	—(CH <sub>2</sub> ) <sub>n</sub> C <sub>6</sub> H <sub>4</sub> OCH <sub>3</sub> - <i>p</i>	4	0.3 ± 0.04 (3)	0.5
M-2357		5	0.8	1.4(?)
M-2334		9	2.6 (2)	0.2

are expressed on an absolute weight basis; in the chart of relative molar activities for the *n*-alkyl series, for example, the peak for the C<sub>9</sub> side chain appears very much more pronounced and there is a less abrupt loss in activity from C<sub>10</sub> on. Both sets of data are subject to considerable experimental error and to the uncertainties of biological variation; some of the *in vivo* activities are based upon uncorrected ED<sub>95</sub> values observed in a single assay, and some of the *in vitro* activities represent the results of a single series of Warburg determinations. Within the rather wide limit of tolerances indicated, it can be concluded from the charts that when the naphthoquinone side chain contains no more than about 10 carbon atoms the relative *in vivo* and *in vitro* activities show considerable correspondence. With an increase in the size of the side chain beyond this limit the *in vitro* activity continues to rise, probably as long as the naphthoquinone sodium salt possesses adequate solubility, whereas the *in vivo* activity either falls off or increases to a lesser extent as the result of increasingly poor absorption from the gut. That members of the arylalkyl series having C<sub>15</sub> to C<sub>21</sub> side chains are surprisingly high in *in vivo* activity may be associated with the greater hydrophilic character of this type of side chain, particularly when it contains a halogen substituent.

The enhancement of both types of activity by a *p*-halo substituent is noteworthy. The activities of *p*-chloro- and *p*-bromophenyl derivatives parallel one another closely over a wide range of activity (0.2 to 7). The following comparisons can be made of the ratio of activity of a *p*-chloro compound to that of the *p*-bromo derivative: *in vitro*, 1.7, 0.9, and 1.0, average 1.2; *in vivo*, 0.4, 1.3, 0.9, and 1.2, average 0.95. Evidently chloro and bromo derivatives possess the same potency on a molar basis, and hence the chloro compounds would be given preference in practical therapy. One comparison of *in vivo* activities indicates that a *p*-iodo derivative corresponds to the chloro and bromo compounds in molar activity; two comparisons are in agreement in showing that *p*-fluorophenyl derivatives have only 40 per cent the molar potency of the chloro, bromo, and iodo analogues.

Although the seven charts of Fig. 1 of relative *in vitro* activity may seem to conform to widely varying patterns, only one of them, that for the *n*-alkyl series, is extensive and complete enough to form a satisfactory basis for judgment. In this series the activity rises slowly and steadily to a peak at C<sub>9</sub> and then increases further to progressively higher peaks at C<sub>11</sub> and C<sub>13</sub>. The curve for the isoalkyl series starts off in a similar manner but extend only through one substantial peak (at C<sub>10</sub>); those for the two cycloalkylalkyl series may represent merely the first parts of the curves of the *n*-alkyl type. The curve for the  $\omega$ -phenylalkyl series, complete from C<sub>9</sub> to C<sub>15</sub>, shows an initial slow rise and then a surge to a possible peak; again there is a suggestion of conformance to the pattern of the *n*-alkyl

series. The *p*-phenoxyphenylalkyl series can perhaps be regarded as one having only two lower members before a  $C_{15}$  peak is reached.

On the assumption that the incomplete charts represent fragments conforming approximately to the general pattern discernible in the *n*-alkyl series, it is understandable why an alternation in the *in vitro* activities of odd and even carbon homologues is observed in some series but not in others. Alternation occurs in the *n*-alkyl series, but only in the region  $C_3$  to  $C_{14}$  and not among the lower members. In the isoalkyl series a striking alternation is observed among the  $C_9$ ,  $C_{10}$ , and  $C_{11}$  derivatives. In the next four series of Fig. 1 the charts are incomplete in the region where alternation could be reasonably expected. The data for the *p*-phenoxyphenylalkyl series cover this favorable region, and striking alternation exists from  $C_{14}$

TABLE II  
Relative Susceptibility to Human Protein Antagonism

Code No.	Side chain	Relative anti-respiratory activity (duck)	$\frac{IC_{50}^{human}}{IC_{50}^{duck}}$
M-1926	$-C_{11}H_{21}-n$	3.1	1.0
M-1928	$-C_{12}H_{23}-n$	2.5	0.71
M-1924	$-C_{13}H_{25}-n$	4.6	0.82
M-2347	$-C_{14}H_{27}-n$	1.9	0.97
M-2338	$-(CH_2)_2C_6H_4OC_6H_5-p$	2.8	0.47
M-2309	$-(CH_2)_3C_6H_4OC_6H_5-p$	4.6	4.1
M-2361	$-(CH_2)_4C_6H_4OC_6H_5-p$	0.9	0.41
M-2345	$-(CH_2)_5C_6H_4OC_6H_5-p$	2.5	0.73
M-384	$-CH_2-4'-Cyclohexylcyclohexyl-cis$	4.6	1.4
M-2329	$-(CH_2)_2-4'-Cyclohexylcyclohexyl-cis$	8.7	3.8
M-2291	$-(CH_2)_3-4'-Cyclohexylcyclohexyl-cis$	7.1	1.3

to  $C_{17}$ . Since the  $C_{21}$  homologue has an odd carbon side chain, similar to the peak  $C_{15}$  and  $C_{17}$  members, it may represent a further peak of the incomplete chart. The extremely potent  $C_{15}$  *p*-chlorophenyl compound may bear a similar relationship to the  $C_{11}$  homologue of its series. Another limited instance of alternation not shown in Fig. 1 is among the three *cis*-4'-cyclohexylcyclohexylalkyl derivatives, whose *in vitro* activities are 4.6, 8.7, and 7.1; in the trans series no alternation was observed. Parallel alternations in *in vivo* activity are apparent with the  $C_8$  to  $C_{10}$  *n*-alkyl compounds and with the  $C_{14}$  to  $C_{16}$  *p*-phenoxyphenylalkyl derivatives, whereas an alternation in the opposite sense is indicated for the three *cis*-4'-cyclohexylcyclohexyl compounds. The alternation in the *in vitro* activities is more profound, and the effect is of so striking a magnitude as to be indicative of a real phenomenon. Perhaps the odd or even character of the side chain determines firmness of binding to proteins.

The experiments reported in Table II were made to see whether alternation could be detected in the antagonistic effect of human plasma proteins. The three series of homologues selected for study all exhibit striking alternation in antirespiratory activity in suspensions in duck serum. In the *n*-alkyl series such differences as were observed in the relative susceptibility to added human serum are within the limit of experimental error. In the other two series a striking and consistent alternation was observed; odd and even homologues vary in susceptibility in some instances by a factor of 10. In each case the compounds that possess the peak potencies are the ones most strongly antagonized by human plasma proteins. The relationship is unfortunate from a practical point of view and demonstrates the importance of considering all available criteria for drug evaluation. That naphthoquinones particularly potent as respiration inhibitors are also highly susceptible to deactivation by specific proteins, presumably by virtue of a strong binding to the protein, suggests that the inhibitory action involves competitive protein binding; the drug action may therefore consist in combination with, and deactivation of, a respiratory enzyme.

#### SUMMARY

1. Comparison of relative *in vivo* activities of 82 naphthoquinones with the relative antirespiratory activities of the compounds in suspensions of parasitized erythrocytes shows that the two manifestations of biological activity are not fully parallel but that the convenient *in vitro* test provides a reliable guide to members of the series likely to possess significant *in vivo* activity.

2. In some of the series investigated, alternations for odd and even carbon homologues are observable both in the *in vitro* activities and in relative susceptibilities to human protein antagonism. Since the homologues of highest potency are those of greatest susceptibility, drug action apparently consists in combination with a respiratory enzyme and resulting deactivation.



# THE NICOTINIC ACID, RIBOFLAVIN, D-AMINO ACID OXIDASE, AND ARGINASE LEVELS OF THE LIVERS OF RATS ON A PROTEIN-FREE DIET\*

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(Received for publication, July 20, 1948)

It is well established that in states of inanition and during periods of restricted protein intake animals suffer a loss of tissue protein, but the significance of this loss with relationship to enzyme activities and vitamin concentrations of tissue has not been clearly elaborated. In this respect, however, Potter and Klug (1) reported that the livers of rats maintained on a diet high in carbohydrate and low in protein or a diet high in fat and low in protein possessed diminished succinoxidase activity and decreased capacity for the oxidation of octanoate and citrate. Axelrod, Swingle, and Elvehjem (2), on the other hand, have reported an increase in the succinoxidase activity of livers from rats maintained on a restricted food intake for 3 weeks.

Recently, in a more extensive study, Miller (3) reported that the activities of a number of enzymes, including catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin, are decreased in the livers of rats fasted for a period of 7 days. The observed loss in enzyme activity apparently paralleled or exceeded the loss of liver protein, and accordingly this author concluded that the decrease in activity represented a loss of enzyme protein *per se*. Similar results were reported by Miller (4) with respect to rats maintained on a protein-free diet.

A more quantitative relationship has been shown to exist between protein intake and liver arginase activity by Lightbody and Kleinman (5). These authors demonstrated that the liver arginase activity was directly related to the protein intake and the length of time the animals were maintained on a particular dietary régime. This relationship was interpreted as expressing an adaptation of the arginase content to a need for the enzyme as determined by the amount of protein which the organ-

\* Aided by a grant from Wyeth, Incorporated, Philadelphia. A preliminary report of this work was presented before the American Society of Biological Chemists, Atlantic City, New Jersey, March 15, 1948 (Seifter, S., Harkness, D. M., Feldman, B., Rubin, L., and Muntwyler, E., *Federation Proc.*, 7, 187 (1948)).

ism was required to metabolize. No attempt was made by these workers to correlate the observed changes with the concentration of liver protein.

A relationship likewise has been shown to exist between protein intake and the liver concentrations of certain B vitamins. Thus, Sarett and Perlzweig (6), Unna and coworkers (7), and Reisen, Schweigert, and Elvehjem (8) have shown that the liver riboflavin is decreased in rats maintained on a low protein diet. On the other hand, Flinn and coworkers (9) observed an increase in the liver concentrations of nicotinic acid, pantothenic acid, pyridoxine, biotin, and vitamin B<sub>6</sub> in chronically undernourished animals.

The present investigation was undertaken to determine (a) the nature of the relationship existing among the enzyme activities, vitamin concentrations, and the protein levels of tissues, and (b) the effect that variations in the diet have on this relationship. More specifically, the study is concerned with the changes which occur in the D-amino acid oxidase and arginase activities and the riboflavin and nicotinic acid concentrations of the livers of rats maintained on a protein-free diet. These particular liver constituents were chosen for study because they include (a) an enzyme which requires a vitamin coenzyme, (b) an enzyme which requires no known vitamin coenzyme, and (c) two vitamins which are known to be components of various enzyme systems.

#### EXPERIMENTAL

Twenty-four male Wistar strain rats, between 16 and 23 weeks of age and weighing between 280 and 313 gm., were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this time the animals were divided into three groups of eight animals each. Four animals in Group A were placed on a protein-free diet for a period of 7 days. In a similar manner four animals in Group B were maintained on the same protein-free diet for 14 days. In Group C, four animals were kept on the protein-free diet for 21 days. In each group the remaining four animals served as pair-fed controls.

The control diet consisted of commercial casein, 20 per cent; sucrose, 65 per cent; corn oil, 10 per cent; Salts 4 (10), 4 per cent; and dried brewers' yeast, 1 per cent. The protein-free diet was similar in composition except that the casein was replaced with an equal amount of sucrose. In addition all animals received daily 2 drops of a vitamin supplement which furnished the following water-soluble vitamins in the amounts indicated: thiamine 25  $\gamma$ , riboflavin 30  $\gamma$ , nicotinamide 25  $\gamma$ , pyridoxine hydrochloride 25  $\gamma$ , calcium pantothenate 200  $\gamma$ , choline chloride 10 mg., and inositol 3 mg. Twice weekly all animals received 2 drops of a cod liver oil-vitamin E supplement which furnished 200 U. S. P. units of vitamin A, 20 U. S. P. units of vitamin D, and 1 mg. of  $\alpha$ -tocopherol.

At the end of the indicated feeding period the animal was sacrificed by a sharp blow on the head; its liver was quickly excised and blotted free of adhering blood, and sampled immediately for the determinations outlined below. Water was determined by drying to constant weight at  $103^{\circ}$ , and fat by weight difference after extraction with ether. Nitrogen was determined on the dry, fat-free residue by the micro-Kjeldahl method. Glycogen content, determined on the livers of some rats as mentioned below, was measured by the method of Deane and his coworkers (11). Nicotinic acid was determined microbiologically with *Lactobacillus arabinosus* as described by Snell and Wright (12). Riboflavin was determined microbiologically with *Lactobacillus casei* as described by Snell and Strong (13). The microbiological procedures were carried out on an extract prepared by autoclaving a weighed portion of liver at 15 pounds for 30 minutes.

Arginase activity was measured on an aliquot of a liver homogenate equivalent to approximately 0.3 mg. of tissue. The method of Van Slyke and Archibald (14) was employed for the determination, the formed urea being determined colorimetrically by the method of Archibald (15). Activity is expressed in Van Slyke-Archibald units.

In order to obtain maximum arginase activity, the liver aliquot was homogenized in 0.05 M  $\text{MnSO}_4$  prepared in 0.9 per cent NaCl. It was found that the arginase activity as determined was dependent upon the length of time that elapsed between the killing of the animal and the initiation of the activation with manganese and also on the duration of the activation period. In order to keep these factors constant, and thus to obtain results on a comparable basis, a strict time schedule was adopted wherein homogenization with the manganous sulfate solution was started 5 minutes after the death of the animal, and activation was allowed to continue for 10 minutes before incubation with the substrate.

D-Amino acid oxidase was determined by a modification of the method described by Rodney and Garner (16). The modified procedure involves the measurement of the amount of keto acid formed by a liver homogenate after incubation with DL-alanine at a concentration of 0.005 M. This method was checked against determinations made by measuring the oxygen uptake under similar conditions, and excellent correlation was found within a fairly narrow range of tissue concentration. The essential steps of the procedure are detailed below.

The test system, contained in a 125 ml. Erlenmeyer flask, consisted of the following materials in the amounts indicated: 0.5 ml. of 0.04 M sodium arsenite prepared in 0.11 M NaCl, 0.5 ml. of 0.04 M DL-alanine, 2.0 ml. of Krebs-Ringer solution from which the calcium had been omitted, and 1.0 ml. of a finely divided, well mixed, liver homogenate containing between 100 and 200 mg. of tissue. A blank determination was prepared in the

same manner except that the DL-alanine was replaced by an equivalent quantity of the Ringer's solution. The remainder of the determination was then carried out by the method of Sealock (17). The air phase above the mixture in the flask was replaced by oxygen, the flask was stoppered, and the system then allowed to incubate at 37° for exactly 6 hours. At the end of this time the reaction was stopped by the addition of 1.0 ml. of 20 per cent trichloroacetic acid, and 5 ml. of water were then added. The solution was filtered and the amount of keto acid formed was deter-

TABLE I

*Water, Fat, Glycogen, and Nitrogen Contents of Livers of Protein-Deficient and Control Rats*

Group	No. of animals	Water	Fat	Glycogen	Nitrogen		
					On basis of fat-free solids	On basis of wet tissue	On basis of solids free of fat and glycogen
		gm. per kg.	gm. per kg.	gm. per 100 gm.	gm. per kg.	mg. per gm.	gm. per 100 gm.
A Protein-free, 7 days	4	708.6 (695-718)	24.2 (18-33)		9.45 (9.0-10.5)	25.20 (23.7-27.8)	
	Pair-fed control	4 697.4 (689-713)	15.7 (11-23)		11.29 (9.9-13.1)	32.25 (29.5-34.6)	
B Protein-free, 14 days	4	687.1 (671-700)	58.1 (31-67)		8.60 (7.2-10.0)	21.85 (18.2-23.7)	
	Pair-fed control	4 698.9 (695-702)	11.5 (9-15)		11.82 (11.1-12.7)	34.14 (32.5-36.0)	
C Protein-free, 21 days	8	668.4 (636-694)	80.0 (45-122)	9.1* (8-10)	9.15 (7.9-10.5)	21.95 (20.8-24.6)	13.90* (12.9-14.9)
	Pair-fed control	8 696.3 (692-703)	16.4 (9-29)	3.8* (2-5)	11.80 (10.4-12.7)	33.59 (30.1-36.8)	13.70* (13.3-14.0)

The figures in parentheses represent the range of the values.

\* Four animals.

mined on 1 ml. of the filtrate by means of a 2,4-dinitrophenylhydrazon procedure. The D-amino acid oxidase activity is expressed in micromoles of pyruvate formed per unit of liver under these conditions.

### Results

Throughout the course of the study the experimental animals consumed an average of 11 gm. of food per day, and, on the average, lost 7 gm. in body weight per week. The animals on the control diet, which were paired to the protein-restricted group, consumed an equal amount of food and gained, on the average, 3 gm. in body weight per week.

The results obtained from the analyses of the livers of the protein-

restricted and control rats for water, fat, and nitrogen are presented in Table I. Maintenance on a non-protein diet, under conditions of adequate caloric intake, resulted in a decrease in the concentration of liver protein as indicated by the amount of nitrogen obtained per gm. of liver. The rate

TABLE II

*Nicotinic Acid and Riboflavin Concentrations and Arginase and D-Amino Acid Oxidase Activities of Livers of Protein-Deficient and Pair-Fed Control Rats*

Group (4 animals each)		Nicotinic acid		Riboflavin		Arginase*		D-Amino acid oxidase activity†	
		$\gamma$ per gm.	mg. per gm. N	$\gamma$ per gm.	mg. per gm. N	units per gm.	units $\times 10^{-3}$ per gm. N	units per gm.	units per gm. N
A	Protein-free, 7 days	102.0	4.06	18.0†	0.701†	86§	3.6§	15.0	596
		(95-104)	(3.8-4.4)	(17-19)	(0.66-0.77)	(76-95)	(3.1-4.0)	(12-20)	(489-805)
	Pair-fed control	152.8	4.75	26.6†	0.824†	434§	15.2§	34.6	1098
		(133-172)	(4.3-5.2)	(22-38)	(0.65-1.13)	(426-503)	(14.4-16.1)	(32-38)	(923-1212)
B	Protein-free, 14 days	79.5	3.64	16.4†	0.734†	138	6.3	11.8	529
		(62-99)	(3.0-4.2)	(13-18)	(0.71-0.78)	(35-263)	(1.9-12.8)	(8-15)	(395-646)
	Pair-fed control	139.1	4.08	35.3	1.037	560†	16.4†	39.2	1154
		(106-172)	(3.3-5.1)	(34-37)	(0.98-1.15)	(429-737)	(13.2-21.8)	(35-41)	(970-1258)
C	Protein-free, 21 days	74.3	3.42	17.8	0.825	103†	4.8†	11.9	529
		(65-81)	(3.1-3.9)	(13-22)	(0.60-1.04)	(54-243)	(2.5-11.6)	(10-15)	(437-653)
	Pair-fed control	147.2	4.58	33.1	1.015	383†	12.1†	43.1	1310
		(125-181)	(3.6-6.0)	(25-44)	(0.84-1.24)	(320-420)	(10.6-13.9)	(34-64)	(980-1802)

\* The unit is defined as the amount of arginase which in 1 minute at 25°, at pH 9.5, and substrate concentration of 0.285 M arginine will decompose 1 micromole of arginine to form 1 micromole of urea (see (14)).

† The activity is expressed here as units which are equivalent to the micromoles of pyruvate formed under the conditions of the assay.

‡ Three animals only.

§ Two animals only.

of decrease was greatest in the 1st week of protein restriction; however, the maximum absolute decrease was already apparent in the animals on the diet for 14 days. The experimental animals maintained for 21 days showed no further loss of liver protein concentration as compared with the 2 week group.

The diminution of liver protein was accompanied by a steady fall in the water content of the liver and a steady rise in the liver fat. As can be

seen, the liver fat increased from about 1.5 per cent in the control animals to 8 per cent in the animals subsisting on the protein-free diet for 21 days.

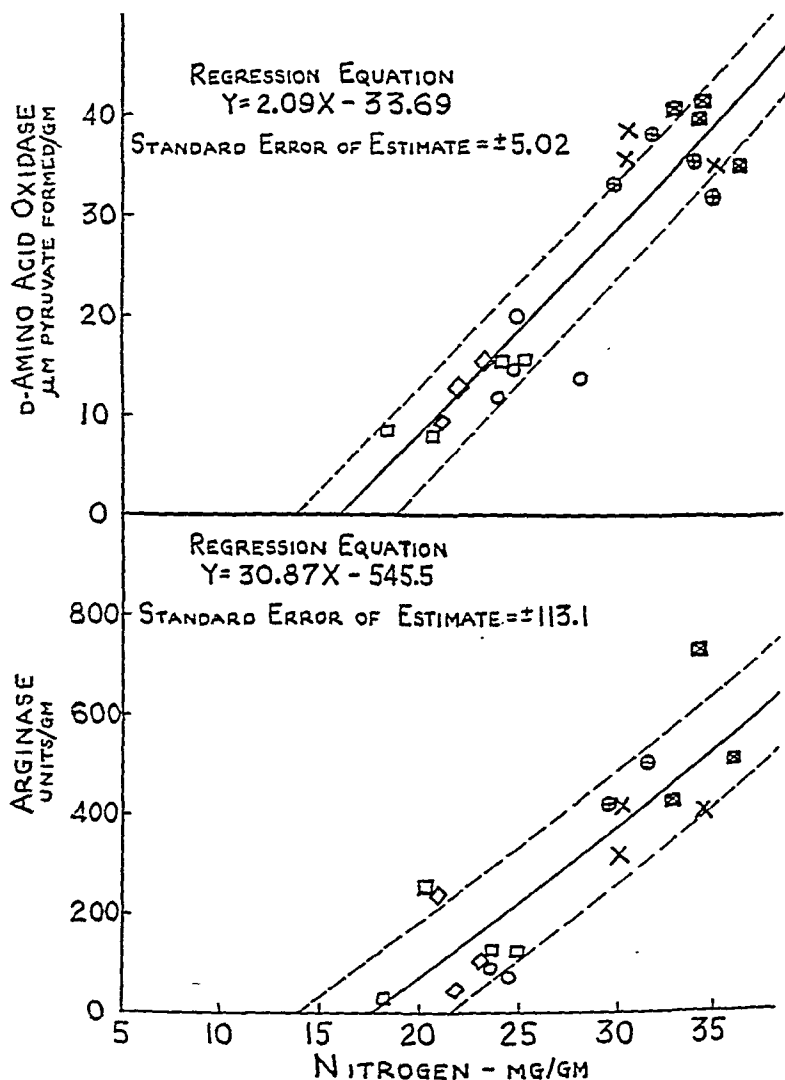


FIG. 1. Liver D-amino oxidase and arginase activities with respect to variations in liver nitrogen content. O, Group A, protein-free diet;  $\ominus$ , Group A, control diet;  $\square$ , Group B, protein-free diet;  $\boxtimes$ , Group B, control diet;  $\diamond$ , Group C, protein-free diet;  $\times$ , Group C, control diet.

This change occurred even though all animals received 10 mg. of choline chloride per day.

The reduction in the nitrogen content of the fat-free liver solids in the groups of animals on the protein-free diet, which becomes apparent when

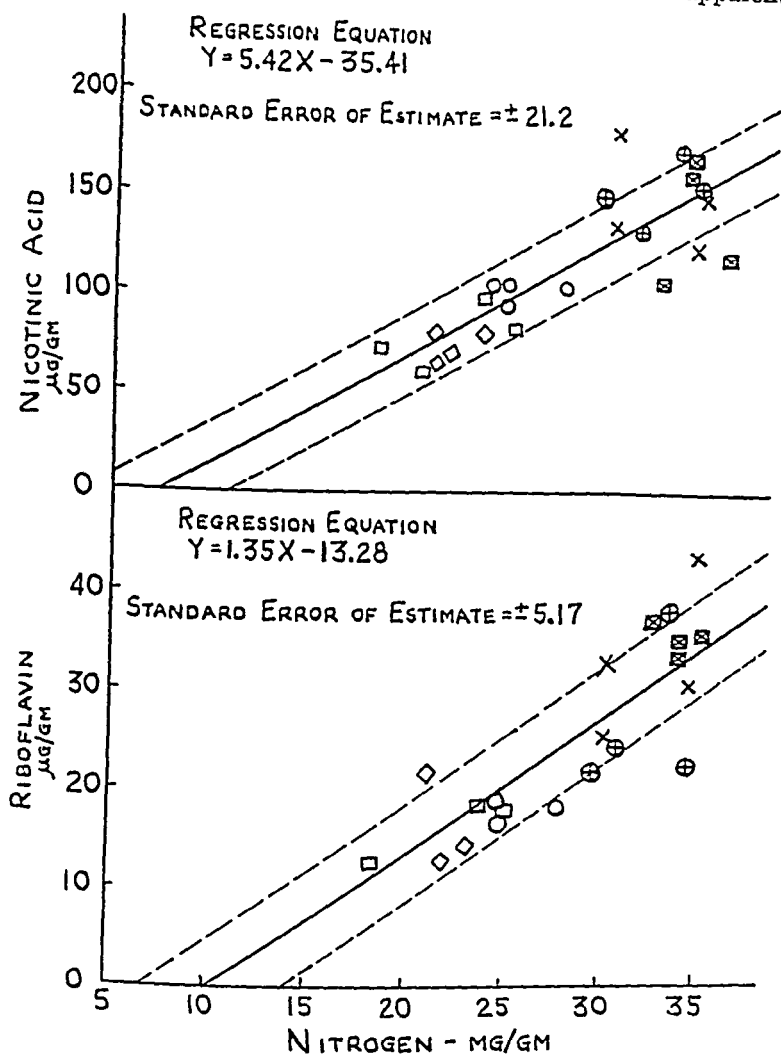


FIG. 2. Liver nicotinic acid and riboflavin concentrations with respect to variations in liver nitrogen content. For interpretation of reference points see the legend to Fig. 1.

comparison is made with the pair-fed controls, was most probably due to an increase in the liver glycogen. In evidence of this, Table I includes the values for liver glycogen of eight additional rats which were maintained on the same dietary régimes as the animals in Group C. The data show

that the animals on the protein-free, high carbohydrate diet had an average liver glycogen content of 9 per cent as compared with the figure of 4 per cent for the control group.

The results obtained from the analyses for riboflavin, nicotinic acid, D-amino acid oxidase, and arginase are presented in Table II. As in the case of the changes in protein concentration of the liver, protein restriction resulted in a decrease in the liver concentration of nicotinic acid and riboflavin as well as in the activities of the two enzymes. The diminution in the amounts or activities of these substances in all cases exceeded the decrease in the concentration of liver protein. This may be seen by comparing the values obtained per gm. of nitrogen in the protein-restricted groups with those obtained in the pair-fed controls. On this basis, the magnitude of decrease was considerably greater in the case of the enzymes than in the case of the vitamins.

The actual levels of the activities of D-amino acid oxidase and arginase were found to be directly related to the concentration of protein in the liver, as is shown in Fig. 1; here is shown the best straight line for the plot of enzyme activities against nitrogen per gm. of liver. From Fig. 1 it can be seen that a decrease in the liver protein concentration was accompanied by a decrease in the activities of these two enzymes. However, the rate of diminution of enzyme activity appears to have exceeded the rate of reduction in the liver protein as evidenced by the failure of the curve to go through the origin upon extrapolation to an enzyme activity of zero.

As is shown in Fig. 2, a similar type of relationship was observed to exist between the concentration of liver protein and the concentrations of riboflavin and nicotinic acid. As in the case of the enzymes, the levels of these two vitamins were directly related to the level of protein in the liver. This relationship was seen to hold, although the intake of these vitamins for all animals was maintained constant at a supposedly adequate level. Also, as was found with respect to the enzymes, the rates of decrease in the concentrations of riboflavin and nicotinic acid in the liver were greater than the rate of reduction in the protein concentration.

#### DISCUSSION

The increase in liver fat which, in the present study, was found to accompany protein restriction in the diet, is similar to the findings of Reisen and coworkers (8) for rats receiving a low protein diet and comparable amounts of choline. That such an increase is not due to a decreased intake of methionine *per se* was shown by these workers. In their experiments, for instance, the incorporation of this amino acid into the low protein diet at a level equivalent to the methionine content of



a 20 per cent casein diet did not cause a lowering of the level of liver fat.

The decrease in the liver concentration of protein encountered in animals on a protein-free diet under conditions of adequate caloric intake is in marked contrast to the protein changes which apparently occurred in the livers of *fasted* rats as observed by Miller (3). For a given enzyme one may take the figure reported by this author for units of enzyme activity per gm. of liver and divide it by the reported figure for units of activity per gm. of liver protein. This result, divided by the factor 6.25, then yields a figure representing the concentration of nitrogen in the particular livers under consideration. Such an analysis of Miller's data shows an increase in the nitrogen concentrations of the livers of the fasted animals (36 mg. of nitrogen per gm. of liver) as compared with the control animals (32 mg. per gm. of liver).

The diminution in the activities of D-amino acid oxidase and arginase with dietary protein restriction is in agreement with the results obtained by Potter and Klug (1) with respect to the capacity of livers obtained from rats which had subsisted on a low protein diet to oxidize succinate, citrate, and octanoate, and, further, is consistent with the results of Miller (4), who found a similar decrease in the activities of five liver enzymes in animals fed a non-protein diet. The changes which have been reported with regard to the enzyme activities of the livers of fasted and undernourished animals (2, 3) would appear to indicate that the mechanisms involved in producing these changes differ from those operating in causing the changes observed in animals restricted only with respect to dietary protein. Furthermore, the direct relationship between enzyme activity and liver protein concentration, as found in the present study, does not necessarily hold in the case of fasting animals. Thus Miller (3) has found that the xanthine dehydrogenase and catalase activities of the livers of fasting animals may be found to decrease, even though appropriate calculations, as explained previously in this discussion, indicate that the protein concentration increases.

Since riboflavin is an integral part of the coenzyme necessary for the activity of D-amino acid oxidase and since the riboflavin concentration in the liver decreased on a non-protein diet, it would appear quite possible that the changes in D-amino acid oxidase activity observed in this study could be due to a decrease in coenzyme concentration. However, since a similar decrease was encountered in the activity of the enzyme arginase which does not require a vitamin cofactor for its activity, it is more likely that the diminution in activity of both enzymes represented a loss in enzyme protein *per se*. In this regard it is interesting to note that Lan

(18) has reported that rats bearing transplanted hepatomas showed a greater decrease in the activity of liver D-amino acid oxidase than of the liver concentration of the coenzyme.

The observed changes in the riboflavin and nicotinic acid concentrations in the liver would appear to indicate that the levels of these vitamins in the liver are independent of the intake over and above a certain minimum requirement, but rather depend largely on the level of tissue protein. In the case of riboflavin this observation is supported by similar findings which have been reported by other investigators (6-8).

The direct relationship which was found to exist between the concentration of liver protein and the levels of riboflavin and nicotinic acid suggests that these vitamins exist in a combined form in the liver and are not present as the free vitamins. Such an observation with respect to nicotinic acid has previously been made by Robinson and his coworkers (19).

#### SUMMARY

The effects of feeding rats a diet free of protein for varying periods of time have been studied with respect to the concentration of protein, riboflavin, and nicotinic acid, and the activities of D-amino acid oxidase and arginase in the livers of these animals.

Maintenance of rats on a protein-free diet caused a decrease in the concentration of liver nitrogen, a decrease in the liver water content, and an increase in the liver fat.

Animals subsisting on a protein-free diet possessed diminished liver arginase and D-amino acid oxidase activities and decreased liver concentrations of riboflavin and nicotinic acid as compared with pair-fed controls. These decreases exceeded the loss in liver nitrogen.

The activities of D-amino acid oxidase and arginase and the levels of riboflavin and nicotinic acid in the liver have been shown to be directly related to the concentration of liver nitrogen under the conditions of these studies.

The significance of these observations is discussed.

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# THE DEGRADATION OF L-LYSINE IN GUINEA PIG LIVER HOMOGENATE: FORMATION OF $\alpha$ -AMINOADIPIC ACID\*

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(Received for publication, June 21, 1948)

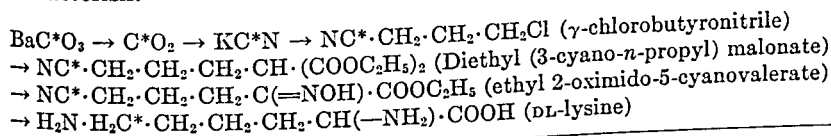
A summary of the little that is known of the metabolism of lysine in animals is as follows: it is indispensable in the diet, its  $\alpha$ -amino group does not participate in reversible transamination reaction *in vivo* (2), neither the L nor D form is attacked by the appropriate amino acid oxidase, certain  $\epsilon$ -nitrogen-substituted derivatives can replace lysine in the diet and their  $\alpha$ -amino groups are oxidized by amino acid oxidases (3, 4), no  $\alpha$ -nitrogen-substituted derivatives yet prepared can substitute for lysine in the diet (4-6).

Partly because so little was known, we have undertaken a study of the metabolism of lysine with the use of  $C^{14}$  as a tracer. The amino acid was synthesized with the isotope in the  $\epsilon$  position and resolved into its L and D isomers. In order to observe in an initial exploration as many aspects of its metabolism as possible, one or the other isomer was made into a mixture with unlabeled amino acids, corresponding to the composition of casein, and incubated with guinea pig liver homogenate under different conditions.

The present communication deals with the finding of  $\alpha$ -aminoadipic acid as a product of the degradation of lysine.

## Preparations

*Synthesis of  $C^{14}$ -Labeled Lysine*—The steps in the synthesis are summarized in the following diagram; the position of the  $C^{14}$  is indicated by an asterisk.



\* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department. A summary of this work has been reported (1).

The  $C^{14}$  used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

$KC^*N$ —309.8 mg. of  $BaC^*O_3$  was converted to  $KC^*N$  by the method of Cramer and Kistiakowsky (7) and Loftfield (8, 9). The strongly alkaline solution obtained after decomposition of the excess potassium was concentrated to 4.7 gm. 90 per cent of the radioactivity was incorporated into the  $KC^*N$ .

$\gamma$ -Chlorobutyronitrile\*—KCN was converted to  $\gamma$ -chlorobutyronitrile by a modification of the procedure previously described (10). To the  $KC^*N$  were added 1.253 gm. of inactive KCN dissolved in 4 ml. of  $H_2O$  and 28 ml. of absolute ethanol. The solution was titrated slowly at  $0^\circ$  to pH 10.8 with 6 N HCl; 3.8 ml. were consumed. The glass electrode was washed with ml. of  $H_2O$  and the washings added to the solution, which was then refluxed with 19.2 gm. of trimethylene chlorobromide with vigorous stirring for  $5\frac{1}{2}$  hours. After cooling, the solution was diluted with 45 ml. of water, and the two layers separated. To the lower layer was added a chloroform extract (12 ml.) of the top layer. The mixture was washed with 10 ml. of a 16 per cent  $CaCl_2$  solution, then with 3 ml. of water, and dried over fused  $CaCl_2$ . The chloroform was removed by distillation at atmospheric pressure, and the residue distilled *in vacuo*; the product obtained at  $89-93^\circ$  and 24 mm. was used for the next step. Yield, 1.535 gm., or 75 per cent.

Diethyl (3-Cyano-*n*-propyl) Malonate\*—From the nitrile, DL-lysine was prepared by a modification of the method of Fischer and Weigert (11). 1.0 gm. of sodium in a Carius tube was dissolved in 10 m<sup>l</sup>. of absolute ethanol. After cooling there were added 13.3 gm. of diethyl malonate, then a mixture of 13.3 gm. of diethyl malonate and 2.74 gm. of chlorobutyronitrile\*, followed by 13.3 gm. of diethyl malonate. The tube was sealed and heated for 15 hours at  $95-98^\circ$ . Ethanol, malonate, and remaining nitrile were removed by steam distillation, the residue extracted with ether, and the ether extract dried over potassium carbonate for 15 minutes, filtered, and distilled at 0.5 mm. Yield, 5.20 gm. (86 per cent); b.p.  $128-131^\circ$  at 0.5 mm.

Ethyl 2-Oximido-5-cyanovalerate\*—0.166 gm. of sodium was dissolved in 2.64 ml. of absolute ethanol and cooled to  $-18^\circ$ . A mixture of 1.13 ml. of ethyl nitrite with 1.628 gm. of cold diethyl (3-cyano-*n*-propyl) malonate\*, cooled to  $0^\circ$ , was then added dropwise into the ethylate solution and washed down with 0.66 ml. of cold absolute ethanol. After standing for 21 hours at  $-15^\circ$  to  $-10^\circ$ , the alcohol was removed at room temperature *in vacuo*. The residue, dissolved in 10 ml. of water, was extracted with 5 ml. of ether, and the ether washed with two 1 ml. portions of water. The combined aqueous solutions were cooled in ice, acidified with 10 per cent sulfuric acid, and extracted with ether. After removal of the solvent *in vacuo* and drying over sulfuric acid, the ester crystallized. Yield, 1.195 gm. (90.5 per cent).

**DL-Lysine\***—In a flask with a reflux condenser 1.252 gm. of ethyl 2-oximido-5-cyanovalerate\* were dissolved in 100 ml. of absolute ethanol, and 10 gm. of sodium added in small pieces as rapidly as possible. After 75 minutes the metal was almost completely dissolved. 10 ml. of water were added, and the solution refluxed for 45 minutes. It was then cooled in ice and acidified by adding 50 per cent  $\text{H}_2\text{SO}_4$  slowly with vigorous stirring. The sodium sulfate was filtered off and washed several times by suspension in 95 per cent ethanol. Filtrate and washings were concentrated *in vacuo*, and the residual alcohol removed by steam distillation. The remaining aqueous solution was made alkaline to phenolphthalein with barium hydroxide, boiled, and the barium sulfate filtered off. The excess barium was removed with carbon dioxide and the filtrate concentrated to a syrup *in vacuo*. The syrup was taken up in ethanol, and a 5 per cent alcoholic solution of picric acid added dropwise with stirring until further addition caused no more turbidity. After standing at  $0^\circ$  overnight the crude picrate was filtered off and washed with cold absolute ethanol and ether. After two recrystallizations, first from 10 ml. and then from 5 ml. of hot water, 0.656 gm. of lysine\* picrate was obtained. Yield, 26 per cent.

The picrate was converted to the hydrochloride by dissolving it in 13 ml. of hot water and 2.3 ml. of concentrated  $\text{HCl}$ . After the solution was cooled, the picric acid was extracted with ether. The aqueous phase, on evaporation *in vacuo* and drying over  $\text{H}_2\text{SO}_4$  and  $\text{NaOH}$ , gave DL-lysine dihydrochloride\* in quantitative yield.

**Resolution of DL-Lysine\***—The DL-lysine was resolved by the carbobenzoxy-aniline-papain method of Bergmann *et al.* (12-14).

**Carbobenzoxy-DL-Lysine\***—0.430 gm. of DL-lysine\* dihydrochloride was dissolved in 2.9 ml. of 2 N  $\text{NaOH}$  and cooled in ice. 1.1 ml. of carbobenzoxychloride and 2.3 ml. of 4 N  $\text{NaOH}$  were added in four portions, and the mixture shaken vigorously for 25 minutes while cooling in ice. After extraction with ether the aqueous phase was acidified with  $\text{HCl}$  and the carbobenzoxylysine taken up with ether. It weighed 0.733 gm. after evaporation and drying *in vacuo*; m.p.  $100-103^\circ$ .

**Carbobenzoxy-L-Lysine Anilide\***—0.730 gm. of carbobenzoxy-DL-lysine\* was dissolved in 1.9 ml. of N  $\text{NaOH}$  and 1.8 ml. of water. To this solution were added 0.46 ml. of aniline, 7.1 ml. of a 0.3 per cent aqueous solution of cysteine hydrochloride, 8.2 ml. of citrate buffer solution (pH 5.0), 16.4 ml. of water, 2.5 ml. of a solution of 0.100 gm. of papain in 2 ml. of water, and 2 ml. of citrate buffer. After incubation at  $40^\circ$  for 19 hours, the L-anilide was filtered off and washed once with 1 per cent potassium bicarbonate solution and thrice with water. Yield, 0.465 gm. Recrystallized from 50 per cent ethanol, it melted at  $121-122^\circ$ .

*Analysis*— $C_{25}H_{31}O_5N_2$  (489.55). Calculated. C 68.70, H 6.38, N 8.59  
 Found. " 68.65, " 6.65, " 8.75

*L-Lysine Dihydrochloride*\*—The anilide was refluxed with 4.5 ml. of 6*N* HCl for 2 hours. After cooling, the mixture was diluted with 30 ml. of water and made alkaline with freshly prepared silver oxide. After the silver chloride and excess silver oxide were filtered off and washed with water, the filtrate was extracted with ether. The clear aqueous phase was concentrated *in vacuo* to 20 ml., acidified with HCl, and allowed to stand for 30 minutes with occasional shaking. After filtration the liquid was evaporated to dryness *in vacuo* over NaOH and  $H_2SO_4$  to give a quantitative yield of lysine dihydrochloride.  $[\alpha]_D^{23} = +15.95^\circ$ , in water;  $c = 5.46$ . Berg (15) reports rotations from  $+15.63^\circ$  to  $+16.55^\circ$  for values of  $c$  from 3.00 to 16.00.

*D-Lysine Dihydrochloride*\*—The filtrate (without washings) of the *L*-anilide was incubated with 25 mg. of papain for 5 days at  $40^\circ$ . After removing the precipitated mixed *L*- and *D*-anilides, the filtrate was acidified with HCl and extracted with ether. 0.243 gm. of carbobenzoxy-*D*-lysine remained upon evaporation of the solvent. After refluxing with 3.0 ml. of 6*N* HCl for 2 hours, extraction with ether, and evaporation of the aqueous phase, 0.130 gm. of *D*-lysine dihydrochloride was obtained. This was recrystallized from ethanol and yielded 99 mg. of purified material.  $[\alpha]_D^{23} = -13.5^\circ$ , in water;  $c = 4.47$ . The rotation corresponds to approximately 92.5 per cent *D* and 7.5 per cent *L* form.

*Analysis*— $C_{25}H_{31}O_5N_2 \cdot 2HCl$ . Calculated. N 12.79, Cl 32.36  
 Found. " 12.44, " 31.82

The total average yield of *L*- and *D*-lysine dihydrochloride obtained in several complete runs was 6 per cent, calculated on the KCN used. In an experiment in which  $BaCO_3$  containing 4 millicuries of radioactivity was used, 230 mg. of *L*-lysine dihydrochloride with a specific radioactivity of 24,000 counts (corrected) per minute per mg. were obtained.

All the radioactivity measurements are expressed as counts (corrected) per minute. They were obtained with standard sample geometry, and corrected for background and resolving time. By means of empirically determined curves of self-absorption loss in different thicknesses of sample, they were corrected to maximum specific activity (16).

*DL- $\alpha$ -Aminoadipic Acid*—DL- $\alpha$ -Aminoadipic acid was prepared by a modification of the method of Sørensen (17). 1.228 gm. of chlorobutyronitrile and 2.060 gm. of diethyl sodium phthalimidomalonate were refluxed in a bath at  $160$ – $165^\circ$ . After 4 hours the alkaline reaction had disappeared, and the excess nitrile was removed by steam distillation. The residue was cooled in ice and washed with water several times by triturating and decant-



ing. It weighed 2.050 gm. after drying. After solution in 12.5 ml. of absolute ethanol, it was heated under a reflux on a steam bath with 11 ml. of 4 N NaOH for 3 hours. 16 ml. of concentrated HCl were then added, the heating continued for 3 hours, and the solution evaporated on the steam bath. The residue was cooled in ice and extracted by repeated washing with ice-cold 33 per cent hydrochloric acid. The solution was filtered through a coarse fritted glass funnel, and evaporated. The residue, weighing 1.206 gm. after drying over  $\text{H}_2\text{SO}_4$  and NaOH, was dissolved in 2.5 ml. of water, filtered into a 10 ml. beaker, and washed with 1.3 ml. of water. The aminoadipic acid was precipitated by titrating to pH 3.1 with 5 N  $\text{NH}_4\text{OH}$ . After standing for 4 hours at  $22^\circ$ , it was filtered off and washed with a few ml. of water, alcohol, and ether. Yield, 0.629 gm. (62 per cent based on diethyl sodium phthalimidomalonate).

The  $\alpha$ -aminoadipic acid was resolved in the same manner as was lysine by the carbobenzoxy-anilide-papain method of Bergmann.

Carbobenzoxy-DL- $\alpha$ -aminoadipic acid, m.p.  $124^\circ$ .

*Analysis*— $\text{C}_{14}\text{H}_{17}\text{O}_6\text{N}$  (295.28). Calculated. C 57.28, H 5.82, N 4.75  
Found. " 57.08, " 5.84, " 4.89

Carbobenzoxy-L-aminoadipic acid anilide, m.p.  $170$ – $171^\circ$ .

*Analysis*— $\text{C}_{20}\text{H}_{22}\text{O}_5\text{N}_2$  (370.39). Calculated. C 64.85, H 5.99, N 7.56  
Found. " 65.56, " 6.04, " 8.30

The L- $\alpha$ -aminoadipic acid melted at  $205^\circ$  with decomposition. Its specific rotation was  $[\alpha]_D^{25} = +33.9^\circ$ , in 6 N HCl;  $c = 5.49$ .

*Analysis*— $\text{C}_8\text{H}_{11}\text{O}_4\text{N}$  (161.17). Calculated. C 44.72, H 6.88, N 8.70  
Found. " 44.83, " 6.81, " 8.65

### Procedure

Guinea pig liver was homogenized in the apparatus of Potter and Elvehjem (18) with a volume of saline solution equal to twice the weight of the liver. The composition of the saline solution was as follows: 0.123 M NaCl, 0.0128 M  $\text{Na}_2\text{HPO}_4$ , 0.005 M KCl, 0.0033 M  $\text{MgSO}_4$ . Unless stated otherwise the pH was 7.5.

The reaction mixture consisted of 2 ml. of homogenate, a sufficient quantity of a mixture of amino acids to provide a final concentration of 1.2 per cent, and 0.01 M  $\alpha$ -ketoglutarate. The amino acid mixture corresponded approximately to the composition of casein. All of the lysine therein contained (10 mg. of the dihydrochloride) was labeled in the  $\epsilon$  position with  $\text{C}^{14}$  (7400 counts (corrected) per mg. per minute). The final volume was 4 ml.; KOH was used for the neutralization.

The reaction mixture was incubated at  $38^\circ$  under oxygen for 6 hours,

after which the pH was adjusted to 5.0. The mixture was then placed in a boiling water bath for 10 minutes, filtered, the coagulated protein thoroughly washed with water, and the washings added to the main filtrate. The non-protein filtrate thus obtained was concentrated *in vacuo*.

### Results

A small fraction of the non-protein filtrate from an experimental run conducted at pH 7.5 was chromatographed on filter paper with phenol and *s*-collidine, and the paper treated with ninhydrin (19). Two radioactive ninhydrin spots were found, one in the position of lysine, the other of glutamic acid. The radioactivity in the latter spot excluded its being glutamic acid, as the probable mechanism for the conversion of lysine to glutamic acid entails cleavage of the radioactive  $\epsilon$ -carbon of lysine. It seemed likely that the substance in question was  $\alpha$ -aminoadipic acid,  $C^*OOH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$ , derived from lysine.

Accordingly,  $\alpha$ -aminoadipic acid was synthesized and chromatographed on filter paper. It gave the same chromatogram as the unknown radioactive substance.

This lead, that the radioactive substance in question might be  $\alpha$ -aminoadipic acid, was followed. The main portion of the non-protein filtrate, from a reaction mixture to which 20 mg. of radioactive L-lysine dihydrochloride had been added, was hydrolyzed by boiling overnight with 20 per cent HCl in order to hydrolyze any peptides present. The latter step was necessary for satisfactory chromatography on Lloyd's reagent (20); it also converted any of the piperidone of  $\alpha$ -aminoadipic acid, which might have been formed, to the straight chain.<sup>1</sup> After removal of the HCl by distillation, the hydrolysate was chromatographed on Lloyd's reagent. The fraction containing all the amino acids except the bases had about 5 per cent of the radioactivity originally added as lysine. It was concentrated to dryness, extracted with ether, and the residue taken up in water and decolorized by boiling with charcoal. The combined filtrate and washings were evaporated to dryness, taken up in 3 ml. of water, and treated with solid  $Ba(OH)_2$  until the pH was 6.0. A small amount of radioactivity was in the precipitate; by far the major portion remained in the solution. Absolute ethanol was added to the latter to a final concentration of 95 per cent. The barium precipitate contained all the radioactivity originally in the solution. The barium was removed with sulfuric acid, the precipitation and resolution repeated three times, and after final removal of the barium the filtrate was concentrated to near dryness. A drop was chromatographed on filter paper. Four ninhydrin spots were obtained; one

<sup>1</sup>  $\alpha$ -Aminoadipic acid like glutamic acid cyclizes. Both forms of both amino acids chromatograph alike on filter paper with phenol and *s*-collidine.

in the glutamic acid region was radioactive, and the other three were in the alanine, aspartic acid, and threonine regions.

Concentrated HCl was added to the main portion of the filtrate and concentrated under a lamp until crystallization set in. About 100 mg. of crystals were collected. They gave a total of 630 counts (corrected) per minute. The mother liquor was found subsequently to have 7650 counts (corrected) per minute. The crystals gave three ninhydrin spots on the filter paper chromatogram; one in the glutamic acid region was radioactive, and the other two were in the aspartic acid and alanine regions.

The radioactive spot certainly contained  $\alpha$ -aminoadipic acid mixed with glutamic acid. We have tried to separate  $\alpha$ -aminoadipic and glutamic acids by chromatography on filter paper with a number of solvent mixtures; none effected a separation.

The hydrochloric acid mother liquor was evaporated to dryness and the dry residue dissolved in 0.1 N HCl. Solid  $\text{Ba}(\text{OH})_2$  was added until the solution was alkaline to phenolphthalein, and then ethanol to a concentration of 75 per cent. 351 mg. of barium salt were obtained, giving a total of 7650 counts (corrected) per minute. The barium was removed with  $\text{H}_2\text{SO}_4$ , the filtrate concentrated to dryness, and the residue dissolved in 0.1 N HCl. The presence of  $\alpha$ -aminoadipic acid in it was determined by crystallization after adding to a portion of the solution, containing approximately 1650 counts (corrected) per minute, 100 mg. of non-radioactive  $\alpha$ -aminoadipic acid as a carrier. The quantity of carrier was about 600 times that of the radioactive form.<sup>2</sup>

The solution was brought to pH 3.1 with ammonia and then concentrated slowly at room temperature under a low vacuum. The crystals which separated out were washed with a small amount of water and then ethanol, dried, and their radioactivity determined. Three recrystallizations were carried out, in the course of which 80 per cent of the carrier was left in the mother liquors. The specific activities of the crystals after each of the four crystallizations were consecutively 11.5, 13.7, 13.2, and 13.8 counts (corrected).

When 13.5 counts (corrected) are taken as the specific activity of the  $\alpha$ -aminoadipic acid after addition of 100 mg. of carrier, 1350 counts (corrected) or 82 per cent of the radioactivity in the solution were in the  $\alpha$ -aminoadipic acid formed from the radioactive lysine added to the reaction

<sup>2</sup> This estimate was made as follows. The lysine dihydrochloride used had a specific activity of 7400 counts (corrected). The equivalent specific activity as  $\alpha$ -aminoadipic acid was, therefore  $(217/161) \times 7400 = 9970$  counts (corrected). A total of 1650 counts in the solution would, then, be given by 0.165 mg. of  $\alpha$ -aminoadipic acid derived from the radioactive lysine added. Its dilution by the carrier would be  $100/0.165 = 606$ .

adipic acid and hydroxy- $\alpha$ -aminoadipic acid. Neither compound was identified with certainty, but there is little room for doubt that they were the amino acids named. The  $\alpha$ -aminoadipic acid amounted to 1.6 per cent and the hydroxy- $\alpha$ -aminoadipic acid to 1.0 per cent of the dry weight of the organism.

Neuberger and Sanger (3, 4) have presented evidence that before the  $\alpha$ -amino group of L-lysine can be attacked by animal tissue enzymes the  $\epsilon$ -amino group must be masked, preferably by acylation. They discussed some possible pathways of the degradation of lysine *in vivo*. In one of them, formation of  $\alpha$ -aminoadipic acid is the first step. The latter surmise is now substantiated by the evidence presented above.  $\alpha$ -Aminoadipic acid, as the first (or one of the first) intermediate in the degradation of lysine, is in accord with the enzymatic findings of Neuberger and Sanger, in that conversion of the  $\epsilon$ -amino group to a carboxyl group is analogous to acylation. It also accounts for the failure of the  $\alpha$ -amino nitrogen of lysine to participate in reversible transamination reactions *in vivo*. It is converted to  $\alpha$ -aminoadipic acid before it yields its  $\alpha$ -amino nitrogen.

We have previously reported (27) evidence of the probable formation of  $\alpha$ -aminoadipic acid from lysine in kidney.

Mitchell and Houlahan (28) have found that  $\alpha$ -aminoadipic acid can replace L-lysine in one lysine-requiring *Neurospora* mutant. The accumulation of large quantities of  $\alpha$ -aminoadipic acid and of hydroxy- $\alpha$ -aminoadipic acid in cholera *Vibrio* points to unusual features of lysine metabolism in that organism which is analogous to those found in mutants of microorganisms.

#### SUMMARY

1. The synthesis and resolution of lysine labeled with C<sup>14</sup> in the  $\epsilon$  position and the synthesis and resolution of  $\alpha$ -aminoadipic acid are described.
2.  $\alpha$ -Aminoadipic acid is formed from L-lysine in guinea pig liver homogenate. D-Lysine is inactive.
3. Over the pH range 7.5 to 9.0, the reaction is fastest at pH 7.5. Boiling destroys the catalytic activity of the homogenate.
4.  $\alpha$ -Aminoadipic acid can be separated from glutamic acid by chromatography on starch.

The authors were assisted by A. A. Dvorsky, D. Eggarter, H. E. Jeffery, G. Oppenheimer, and A. Tollestrup.

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# THE DEGRADATION OF $\alpha$ -AMINOADIPIC ACID IN GUINEA PIG LIVER HOMOGENATE\*

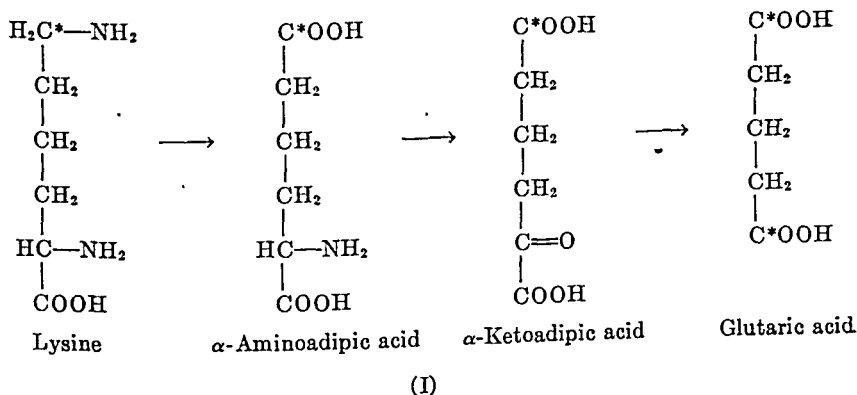
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(Received for publication, June 21, 1948)

In continuation of our study of the metabolism of L-lysine,  $\alpha$ -aminoadipic acid, which is formed from lysine in guinea pig liver homogenate (1), was synthesized with  $C^{14}$  in the  $\epsilon$ -position. The metabolism of the latter compound was followed by search for the radioactive tracer among the probable metabolic products. Two have been identified,  $\alpha$ -ketoadipic and glutaric acids.

The accompanying diagram (I) indicates one of the pathways of the catabolism of lysine. The asterisk indicates the position of the labeled carbon.

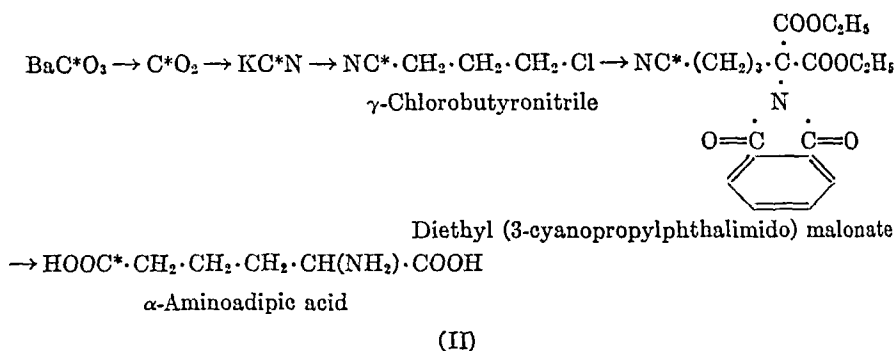


## Preparations

A summary of the synthesis and resolution of  $\alpha$ -aminoadipic acid labeled with  $C^{14}$  in the  $\epsilon$  position is shown in diagram (II). The radioactive carbons are marked with an asterisk.

\* This work is part of that done under contract with the Office of Naval Research, United States Navy Department. It was reported at the meeting of the American Society of Biological Chemists, March 15-19, 1948.

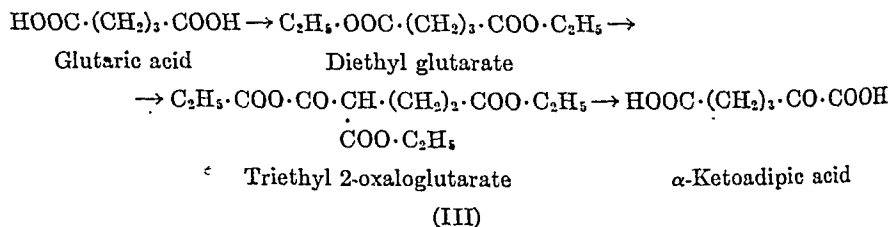
The  $C^{14}$  used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.



The details of the preparation have been described in a previous communication (1).

89.3 mg. of  $\text{BaC}^{14}\text{O}_3$  containing 0.625 millicurie of  $\text{C}^{14}$  yielded 0.127 gm. of pure L- $\alpha$ -aminoadipic acid, giving 14,450 counts (corrected) per mg. per minute.

*α-Ketoadipic Acid*—Diagram (III) is a summary of the synthesis of *α*-ketoadipic acid.



Diethyl glutarate was prepared by the method of Locquin and Elghozy (2), and triethyl 2-oxaloglutarate by the method of Gault (3). The latter compound was decarboxylated with HCl. After removal of the HCl *in vacuo* spontaneous crystallization set in. The crude product was purified by solution in warm ether and reprecipitation by the addition of an equal volume of petroleum ether. The light orange crystals had the following composition.

$C_6H_4O_5$  (160.06). M.p. 124°. Calculated, C 44.98, H 5.04; Found, C 45.12, H 5.04

### Procedure

The preparation of the guinea pig liver homogenate and the saline solution are described in a previous communication (1).

In a representative experiment 2 ml. of homogenate containing 0.66 gm. of liver (wet weight) and 2 ml. of saline solution containing a mixture of 5 mg. of L- $\alpha$ -amino adipic acid (14,450) counts (corrected) per minute per mg.) and 5 mg. of non-radioactive L- $\alpha$ -amino adipic acid were added to



each of four 20 ml. beakers. Two were immediately brought to pH 5.0 and boiled for 10 minutes, the coagulated protein extracted with water, and the non-protein filtrates and washings combined. The other two beakers were incubated at pH 7.4 under oxygen at 38° for 6 hours, then deproteinized by boiling at pH 5.0, and their non-protein filtrates combined.

### Results

The non-protein filtrates were cleared by boiling with charcoal, acidified with hydrochloric acid to a concentration of 0.1 N, and then extracted with ether. After the ether was evaporated, the residue was taken up in 3 ml. of water; 100 mg. of non-radioactive  $\alpha$ -ketoadipic acid and 91 mg. of phenylhydrazine hydrochloride dissolved in 2 ml. of water were added. The oil,  $\alpha$ -ketoadipic acid phenylhydrazone, which settled out first, crystallized in 2 hours. The recrystallization procedure was as follows: 2 ml.

TABLE I  
*Specific Radioactivity of  $\alpha$ -Ketoadipic Acid Phenylhydrazone  
after Successive Recrystallizations*

No. of crystallizations	Counts (corrected) per mg. per min. of phenylhydrazone from	
	Reaction mixture at zero time	Reaction mixture after 6 hrs. incubation
1	1.8	10.1
2	1.0	3.1
3	0	2.7
4	0	2.6
5	0	2.7
6	0	2.8

of water were added to the crystals, and the suspension brought to boil and then treated with ethanol dropwise until all the crystals dissolved. The solution was then cooled, the crystals collected, and their specific radioactivity determined.

Table I gives the specific radioactivities of the phenylhydrazone samples obtained from the reaction mixtures at zero time and after 6 hours incubation. The figures show that by the second recrystallization all the radioactivity had been removed from the phenylhydrazone obtained from the zero time reaction mixture, and that constant specific activity had been attained in the case of the phenylhydrazone obtained after 6 hours incubation. The counts in the first and second crystallizations probably arose from  $\alpha$ -aminoadipic acid, and, in the case of the 6 hour reaction mixture, also from glutaric acid arising from degradation of the  $\alpha$ -ketoadipic acid formed from the added  $\alpha$ -aminoadipic acid.

The melting point of the radioactive phenylhydrazone after the fifth crystallization was 141–142°, and of a mixture with an authentic sample 141–142°. Gault (3) reported the melting point as 141°. After the final recrystallization, 15 mg. of the phenylhydrazone remained. 90 per cent of the added  $\alpha$ -keto adipic acid was, therefore, left in the mother liquors.

These data prove that the  $\alpha$ -amino adipic acid was oxidatively deaminized to  $\alpha$ -keto adipic acid.

The rate of formation of  $\alpha$ -keto adipic acid from  $\alpha$ -amino adipic acid can be calculated from the value of the constant specific activity of the  $\alpha$ -keto adipic acid phenylhydrazone obtained from the reaction mixture incubated for 6 hours. The specific activity of the phenylhydrazone was 2.7 counts (corrected) per mg. per minute. 100 mg. of  $\alpha$ -keto adipic acid, equivalent to 156.3 mg. of its phenylhydrazone, were added as a carrier. The weight of the radioactive form being neglected, the total  $\alpha$ -keto adipic

TABLE II  
*Specific Radioactivity of Barium Glutarate (Dried at 100°) after Successive Reprecipitations*

No. of precipitations	Counts (corrected) per mg. per min. of barium glutarate from	
	Reaction mixture at zero time	Reaction mixture after 6 hrs. incubation
1	0	1.50
2	0	1.33
3		1.30
4		0.95
5		1.01
6		1.01

acid in the solution contained  $156.3 \times 2.7 = 422$  counts (corrected). 144,500 counts (corrected) were added originally as 20 mg. of  $\alpha$ -amino adipic acid. .029 per cent of the latter was found as the keto acid, or 0.058 mg. Expressed as a  $Q$  value,<sup>1</sup> this rate is 0.005. It is about one-twelfth that of the formation of  $\alpha$ -amino adipic acid from L-lysine (1).

In another experiment similar to the preceding, the non-protein filtrate was examined for evidence of formation of glutaric acid. The procedure was the same as before to the stage after evaporation of the ethereal extract of the non-protein filtrate. 100 mg. of non-radioactive glutaric acid were added as a carrier in the subsequent crystallization. The mixture was taken up in 3 ml. of water and treated with saturated barium hydroxide

<sup>1</sup>  $Q$  is the rate of change of the substance in question expressed as if it were a gas in c.mm., at standard temperature and pressure, per mg. of dry weight of tissue used per hour.

solution to pH 9.0; the final volume was 5 ml. No precipitation occurred. 10 ml. of 95 per cent ethanol were then added. The precipitate of barium glutarate pentahydrate was filtered off. 96 per cent of the water of hydration was driven off by drying at 100°, and the specific radioactivity of the compound was determined.

The solution in water and precipitation with ethanol were repeated five times. Table II gives the specific radioactivities of the barium glutarate samples obtained as above from the reaction mixtures at zero time and after 6 hours incubation. After the final reprecipitation 30 mg. of barium salt (as pentahydrate) remained, corresponding to 11 per cent of the original glutaric acid added.

Analysis of the radioactive barium salt after the last precipitation gave, on an anhydrous basis, 51.3 per cent of barium (calculated, 51.4 per cent).

These data prove that glutaric acid was one of the products formed in the liver homogenate from the added  $\alpha$ -aminoadipic acid. There can be little doubt that  $\alpha$ -ketoadipic acid was formed first, and that the glutaric acid arose by its oxidative decarboxylation.

The calculation of the rate of formation of glutaric acid from the added  $\alpha$ -aminoadipic acid is as follows: the final constant specific activity of the barium glutarate was 1.0 count (corrected) per mg. per minute; 100 mg. of glutaric acid, equivalent to 202.5 mg. of anhydrous barium glutarate, were added as carrier; the glutaric acid formed from the added  $\alpha$ -aminoadipic acid contained, therefore, 202 counts (corrected) per minute; 144,500 counts were contained in the  $\alpha$ -aminoadipic acid; 0.14 per cent of the latter was, therefore, found as glutaric acid. The  $Q$  value is 0.0024.

Of the two successive oxidative steps in the degradation of  $\alpha$ -aminoadipic acid, deamination followed by decarboxylation, the latter is probably the faster. 0.43 per cent of the added  $\alpha$ -aminoadipic acid was found in the above two degradation products. This figure represents the rate of its deamination. The corresponding rate of decarboxylation was 0.14/0.43 or 33 per cent.

#### SUMMARY

1. The synthesis of  $\alpha$ -ketoadipic acid is described.
2. In guinea pig liver homogenate  $\alpha$ -aminoadipic acid is oxidatively deaminized to  $\alpha$ -ketoadipic acid and the latter is oxidatively decarboxylated to glutaric acid.
3. The deamination of  $\alpha$ -aminoadipic acid is much slower than its formation from L-lysine. The decarboxylation of  $\alpha$ -ketoadipic acid is faster than the deamination of  $\alpha$ -aminoadipic acid.
4. The foregoing evidence was obtained with the use of lysine and of  $\alpha$ -aminoadipic acid labeled with  $C^{14}$  in the  $\epsilon$  position.

The elementary analyses were carried out by Dr. G. Oppenheimer. The authors were assisted in this work by A. A. Dvorsky, D. Eggarter, H. E. Jeffery, and A. Tollestrup.

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# A STUDY OF CONDITIONS FOR KJELDAHL DETERMINATION OF NITROGEN IN PROTEINS

## DESCRIPTION OF METHODS WITH MERCURY AS CATALYST, AND TITRIMETRIC AND GASOMETRIC MEASUREMENTS OF THE AMMONIA FORMED

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(Received for publication, July 15, 1948)

The publications on Kjeldahl's method for determining nitrogen that have appeared since Kjeldahl's (1) original paper in 1883 perhaps outnumber those on any other analytical method in the same period of time.<sup>1</sup> The diversity of papers is attributable to the immense usefulness of the method, to its need for modifications for applications to various types of organic and inorganic compounds, and to the search for catalysts to provide such modifications and to accelerate the digestion. Kjeldahl himself used a digestion mixture of sulfuric and phosphoric acids which he found was adequate for many organic substances. For alkaloids, however, he found the addition of an oxidizing agent necessary and added potassium permanganate crystals to the hot concentrated digest.

The substances that have been added to the sulfuric acid digest may be divided into three classes: (1) Potassium sulfate to increase the boiling point and thereby accelerate the digestion process. (2) Oxidizing agents. The requirement for these is that they must assist the digestion of organic compounds without destroying any of the ammonia formed. Permanganate was thus used by Kjeldahl. At present the oxidizing agents in most general use are hydrogen peroxide and potassium persulfate. (3) Metallic catalysts and other substances that act as accelerators. Phosphoric acid employed by Kjeldahl is such an accelerator; although its boiling point is lower than that of concentrated sulfuric acid, the presence of phosphoric acid makes digestion go faster. Phosphoric acid has the disadvantage of etching the digestion flask. The metallic catalysts used to accelerate digestion include mercury, copper, and selenium.

<sup>1</sup> The early history of the Kjeldahl procedure from 1883 to 1892 has been fully presented by Vickery (2), and reviews by Friedrich (3) in 1933 and Bradstreet (4) in 1940 give bibliographies.

Potassium sulfate to accelerate the digestion by raising the boiling point was introduced by Gunning (5) in 1889. Mercury was introduced by Wilfarth (6) in 1885. Arnold (7) in 1886 used sulfuric acid with both copper and mercury as catalysts. The Gunning-Arnold procedure was thus named by Arnold and Wedemeyer (8) who recommended a digestion mixture containing both the potassium sulfate introduced by Gunning and the copper and mercury introduced by Arnold. In this country the so called Gunning-Arnold method used as a standard macro-Kjeldahl procedure for several decades has employed a mixture of sulfuric acid, copper sulfate, and potassium sulfate, with or without mercury.

In testing the various modifications of the Kjeldahl method for pure organic substances the results have commonly been compared with the theoretical nitrogen contents of the substances or with the values obtained by the classic Dumas combustion. In applying the various Kjeldahl procedures to protein material, however, theoretical nitrogen values are not available and in most instances Dumas combustions have not been used for comparison, the criterion for satisfactory results being comparison with maximum nitrogen values obtained by other Kjeldahl procedures taken as standards.

The work in the present paper grew out of the observation that when dried preparations of plasma proteins were analyzed for nitrogen by the Campbell-Hanna (9) Kjeldahl procedure, in which a mixture of sulfuric and phosphoric acids with copper and selenium as catalysts is employed and digestion is continued for 12 minutes after clearing, results were several per cent lower than by the Dumas dry combustion. A survey of various digestion mixtures, including metallic catalysts, persulfate, and peroxide, resulted in the conclusion that none of the catalysts that have hitherto been used except mercury would give within a reasonable period of time a nitrogen yield from proteins equal to that of the Dumas combustion.

Presumably the reason why mercury has been little used in routine macro- or micro-Kjeldahl procedures in current use is the added step in the analysis made necessary by the fact that when alkali is added to a mercury-containing solution of ammonia, as a preliminary to distillation of the ammonia, a considerable fraction of the ammonia is bound by the mercuric oxide precipitated and cannot be liberated by boiling. To prevent low results from this cause it has been customary to add either a sulfide, or thiosulfate, which liberates sulfide, in order to precipitate the mercury. The addition of sulfide to the acid solution of digest causes an unpleasant evolution of hydrogen sulfide, and the heavy precipitate of mercuric sulfide increases the tendency to bumping during digestion. These unpleasant features can be avoided by the use of zinc dust to reduce the mercuric

oxide to metallic mercury during the distillation. This device was introduced in 1892 by Böttcher (10) and was used by Arnold and Wedemeyer (8), but apparently has since been overlooked. The zinc dust not only reduces the mercuric oxide to metallic mercury but also, by providing an evolution of fine bubbles of hydrogen gas, causes the boiling during digestion to proceed very smoothly and with minimum danger of bumping.

Sörensen and Andersen (11) studied the application of the Kjeldahl procedure to lysine and found that addition of mercury to the digest was necessary for accurate results. They concluded that any Kjeldahl procedure applied to proteins or their derivatives should be checked by comparison with the Gunning-Arnold method. Miller and Houghton (12) confirmed the necessity of mercury in analysis of lysine. Pregl and Roth (13) recommended the use of mercury in analyses of protein material, but did not emphasize the necessity of the addition.

In the present paper details of a procedure are given for use of a mercury-containing digest with three types of Kjeldahl analyses: (1) macroanalyses, (2) microanalyses with distillation and titration of the ammonia, and (3) microanalyses with gasometric determination of the ammonia by the hypobromite reaction.<sup>2</sup>

For determination of the ammonia in the distillate in the macromethod we have found the procedure of Meeker and Wagner (14) advantageous, compared with the older method of collecting the distilled ammonia in a solution of excess standard acid and titrating back the excess with standard alkali. In the Meeker and Wagner procedure the ammonia is distilled into boric acid solution and is titrated by adding standard sulfuric acid to the distillate until the pH is lowered to that of a control standard with boric acid alone. This procedure eliminates the necessity of using standard alkali. For titrations in the micromethod, however, we have found that routine analyses are more accurate when the older procedure of distilling into excess standard acid is employed, with back titration with alkali. For the small amounts of ammonia present in the microanalyses this procedure gives a sharper end-point than the titration in the presence of boric acid. In the gasometric micromethod the hypobromite procedure for ammonia described by Van Slyke and Kugel (15) is employed, the mercury being first removed by boiling for a moment with zinc dust.

<sup>2</sup> A photometric microprocedure, in which the same digestion mixture is used, has been employed by Dr. Howard Eder in this laboratory and will be reported by him later. After digestion the acid digest is diluted and treated with a small amount of  $\text{Na}_2\text{S}$ . The excess  $\text{H}_2\text{S}$  is removed by boiling for a minute or two, and the ammonia is determined by nesslerizing an aliquot of the clear supernatant.

## METHODS

*Macro-Kjeldahl**Special Apparatus Required*

A still with glass condenser tubes.<sup>3</sup>

Glass spoons to measure 10 gm. of  $K_2SO_4$  and 2 gm. of zinc dust. These spoons are of the type used by Van Slyke and Folch ((17) Fig. 4) and described in detail by Van Slyke, Hiller, Weisiger, and Cruz (18).

*Reagents*

*Powdered potassium sulfate*, ammonia-free.

*Mercuric sulfate solution*. Dilute 12 ml. of concentrated  $H_2SO_4$  to 100 ml. with water, and dissolve 10 gm. of red mercuric oxide in this solution.

*Concentrated sulfuric acid*, C.P.

*Zinc dust* (not granulated zinc), ammonia-free.

*Concentrated sodium hydroxide solution*, approximately 18 N.

*Boric acid*, 4 per cent solution.

*Sulfuric acid*, N/14 solution.

*Indicator*. The mixed indicator described by Meeker and Wagner (14) or a 0.1 per cent solution of brom-cresol green in 95 per cent ethyl alcohol.

*Procedure*

Into 500 ml. Kjeldahl flasks measure the samples to be analyzed, add to each 10 gm. of potassium sulfate, 10 ml. of the mercuric sulfate solution, and 20 ml. of concentrated sulfuric acid. Digest over a low flame until frothing ceases and water has been driven off, then with "subboiling" (*i.e.*, heating just under the boiling point, so that there is an occasional slight ebullition, as originally recommended by Kjeldahl (1)), which is continued for 2 hours after clearing. Cool, add 250 ml. of water, and after cooling add 2 gm. of zinc dust and 50 ml. of 18 N NaOH. Distil into 500 ml. receivers, each containing 50 ml. of 4 per cent boric acid. After 10 or 15 minutes distillation all the mercuric oxide is reduced to metallic mercury, which amalgamates with the zinc, and the solutions become clear. Distillation is continued until about 200 ml. of distillate are collected, or slight bumping begins.

For titration of the ammonia in the distillate add either 5 drops of the mixed indicator of Meeker and Wagner (14) or 0.3 ml. of 0.1 per cent alco-

<sup>3</sup> Some of the metallic mercury formed by action of the zinc distils into the condensers, and, if the condensing tubes are of block tin, they are eventually destroyed by amalgamation with the mercury. The importance of using glass condensers in place of the usual metal condensers in Kjeldahl stills, when mercury is in metallic form during distillation, has been pointed out by Andersen and Jensen (16) in their very thorough paper on the Kjeldahl method.



holic brom-cresol green solution and titrate with the  $N/14$  sulfuric acid till the color matches that of a control flask. The control is prepared by measuring into a flask of the type used as receiver 50 ml. of boric acid, indicator solution, and enough water to make the volume equal to that in the receiver containing the distillate.

*Blank* analyses are performed with all the reagents used.

### Calculation

$$T - B = \text{mg. N in sample analyzed}$$

$T$  indicates the ml. of  $N/14$   $H_2SO_4$  used in titration of the distillate,  $B$  the ml. used in titrating the blank.

### Titrimetric Micro-Kjeldahl

#### Apparatus

Glass spoons to measure 0.5 gm. of  $K_2SO_4$  and 0.2 gm. of zinc dust ((17) Fig. 4; (18)).

Funnel to deliver  $K_2SO_4$  into the bottom of the digestion tube. This should have a stem 12 cm. long and 1 cm. in diameter. It can be made from glass tubing by flaring one end into a funnel.

Test-tubes of Pyrex glass, 22 to 25 by 200 mm. for digestion.

Erlenmeyer flasks of 125 ml. capacity, to serve as receivers.

Burette, 10 ml. calibrated.

Calibrated pipettes, 1, 2, 5, and 10 ml.

Micro digestion rack.

Apparatus for steam distillation of ammonia from the digests.<sup>4</sup>

#### Reagents

The  $K_2SO_4$ , concentrated  $H_2SO_4$ , mercuric sulfate solution, and zinc dust, described for the macroanalysis.

<sup>4</sup> A convenient micro steam distillation apparatus is the type described by Parnas and Wagner (13, 19) in which the contents of the distilling flask are removed automatically by suction at the end of each distillation, and in which no burner is used under the distilling flask. The distilling flask devised by Parnas and Wagner (13), as described in the catalogues of the laboratory supply companies is good, except that in the round bottomed distilling flask the end of the bent inner tube is placed out 0.5 cm. too high to remove the solid particles by suction when zinc dust is added. The end of the inner tube should approach that section of the rounded portion of the flask which is the bottom when placed at the angle used for distillation. An ideal flask for use with zinc dust is one in which the bottom is cone-shaped, with a straight (unbent) steam inlet ending within a few mm. of the bottom. Distilling flasks having these requirements for use with zinc dust were supplied by E. Shlett and Son, 220 East 23rd Street, New York. The condenser should be of glass, not metal, when mercuric sulfate is used as catalyst.

*Alundum chips*, black (Norton's No. 14).

*Sodium hydroxide*, approximately 10 N solution. Dissolve 400 gm. of NaOH in water and dilute to 1 liter.

*Standard 71.4 mM ammonium chloride solution*, to be used for checking the micro-Kjeldahl procedure. Dissolve 0.382 gm. of  $\text{NH}_4\text{Cl}$ , sublimed "analytical reagent" grade, in water, and dilute to 100 ml. 1 ml. contains 1 mg. of nitrogen.

*Acetate buffer* with pH 5, 0.2 M. To 1 liter of 0.2 M sodium acetate (27.22 gm. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  per liter) add 427 ml. of 0.2 N acetic acid (standardized by titration against 0.1 N NaOH with phenolphthalein indicator).

*Alizarin red indicator solution*, 0.1 per cent in water.

*Control flask for titration end-point*. Measure into a 125 ml. Erlenmeyer flask 7 ml. of the 0.2 M acetate buffer, 63 ml. of distilled water, and 0.8 ml. of 0.1 per cent alizarin red indicator solution. This control flask should be made up fresh at least every 3 days, or oftener if molds grow in the solution.

*0.01428 N (N/70)  $\text{H}_2\text{SO}_4$* , made by dilution of standard 1 N or 0.1 N  $\text{H}_2\text{SO}_4$  (14.28 or 142.8 ml. respectively to 1 liter).

*0.01428 (N/70) NaOH solution*. The solution is kept in a heavily paraffined bottle protected against atmospheric  $\text{CO}_2$  by a soda lime tube. The solution is standardized *daily* by titration against 10 ml. portions of the 0.01428 N  $\text{H}_2\text{SO}_4$  with the same pH and volume at the end-point described below for titration of distilled ammonia.

### *Procedure for Titrimetric Microanalysis*

*Digestion*—Into Pyrex glass tubes 22 to 25 by 200 mm. measure 0.5 gm. of  $\text{K}_2\text{SO}_4$ . (For the determination of albumin in a filtrate when the globulin has been precipitated with sodium sulfate, no  $\text{K}_2\text{SO}_4$  need be added.) Samples containing 0.2 to 2 mg. of nitrogen are added, followed by 0.5 ml. of the mercuric sulfate solution, 1 ml. of concentrated sulfuric acid, and three or four pieces of alundum. The mixture is boiled gently over a micro burner until the water is boiled off; then the flame is increased slightly so that the concentrated digest is constantly boiling with *slight* motion. When entirely clear, gentle boiling is continued for 30 minutes. As soon as the tubes are sufficiently cool, about 2 minutes, but *before* the contents solidify, wash down the sides of the tubes with 3 ml. of water.

*Distillation*—Each distilling apparatus should be tested for the volume of distillate required under the conditions used for distillation, to give theoretical results with a standard ammonium chloride solution.

Before each series of distillations the distilling apparatus is steamed out for 30 minutes. Before each digest is transferred from the digestion tube to the distilling flask, the lip of the digestion tube is greased lightly with a little vaseline to avoid loss during transfer and washing. The contents of

the tube are washed into the distilling flask with four portions of 1.5 to 2 ml. each of water. The sides of the tube are rinsed down with each portion. While the third washing is in the funnel of the Parnas distilling apparatus, add to it, in the funnel, 0.2 gm. of zinc dust. Deliver the mixture of washing and zinc dust into the distilling flask and follow it with the fourth washing. Deliver 5 ml. of 10 N NaOH into the distilling flask and distil into 10 ml. of 0.01428 N  $H_2SO_4$  with the tip of the condenser below the surface of the acid. Distil a volume found to be required by the test with standard ammonium chloride solution as described above, then lower the receiving flask so that the tip of the condenser is above the surface of the acid, and distil 1 minute longer. Wash the tip of the condenser into the flask with a few drops of water.

*Titration*—To each distillate add 0.8 ml. of 0.1 per cent alizarin red indicator solution and titrate with 0.01428 N NaOH from a 10 ml. burette till the color matches that of the acetate buffer solution in the control flask. The final volume should approximate that in the control flask.

Blank analyses should be done frequently; the entire procedure should be followed through.

### Calculation

$$\text{Mg. N in sample analyzed} = 0.2 (B - T)$$

$B$  = ml. of 0.01428 N NaOH required in the back titration of blank analyses;  
 $T$  = ml. of 0.01428 N NaOH required in the titration of the distillate.

### Gasometric Micro-Kjeldahl

#### Apparatus

The Van Slyke-Neill manometric apparatus (20, 21).

The glass spoons, funnel, and Pyrex digestion tubes described for the titrimetric microanalysis.

#### Reagents

The  $K_2SO_4$ , concentrated  $H_2SO_4$ , mercuric sulfate solution, and zinc dust, described for the macroanalysis.

*Alundum chips*, black (Norton's No. 14).

*Hydrochloric acid*, approximately 0.01 N.

*Sulfuric acid*, approximately 3.5 N (100 ml. of concentrated  $H_2SO_4$  diluted to 1 liter with water).

*Brom-thymol blue*, 0.4 per cent.

*Sodium hydroxide*, approximately 10 N.

*Bromine solution*. 60 gm. of KBr are dissolved in 100 ml. of water. 2.5 ml. of bromine are dissolved in the KBr solution.

### Procedure

*Digestion*—Digestion is carried out as described for the micro titrimetric procedure.

*Treatment of Digest*—After cooling the digest for 2 minutes, 3 ml. of water and 0.2 gm. of zinc dust are added, and the contents of the digestion tube are heated till they begin to boil, to accelerate amalgamation of zinc and mercury. The mixture is then cooled, a drop of 0.4 per cent brom-thymol blue is added, and the solution is neutralized, as described by Van Slyke (22), by adding from a pipette 10 N sodium hydroxide, a drop at a time, with occasional cooling of the tube in cold water. Addition of alkali is continued until the mixture becomes alkaline to the indicator. 3.5 N sulfuric acid is then dropped in until the color just changes back to acid, in order to prevent loss of ammonia during subsequent deaeration.

*Transfer of Digest to Van Slyke-Neill Chamber*—The digest solution, after the above treatment, is decanted into the cup of the Van Slyke-Neill blood gas apparatus and the volume is noted. The solution is then drawn down into the chamber. Into a graduated pipette is drawn enough 0.01 N HCl to make the total volume of the solution up to 10 or 11 ml., and this HCl is used in three portions to rinse into the gas apparatus the drops of solution adherent to the walls of the digestion tube and to the zinc-mercury amalgam in the bottom of the tube. Each portion is used to rinse the walls of the digestion tube, then the walls of the cup of the gas apparatus, and is then drawn down into the chamber.

*Gasometric Determination of Ammonia*—The cock of the gas chamber is sealed with a drop of mercury, the chamber is evacuated, and is shaken 2 minutes to extract the air from the solution. The extracted air bubble is ejected. The extraction is repeated, and the slight air bubble obtained is ejected.<sup>5</sup> Then 1.25 ml. of 10 N NaOH are placed in the cup of the chamber, and 0.75 ml. of the bromine solution is added and mixed with the alkali. 1.5 ml. of the mixture are run into the chamber, and the cock is sealed with mercury. The chamber is then evacuated and shaken 3 minutes.

*Measurement of Evolved  $N_2$* —The gas volume is reduced to 2 ml. and the reading,  $p_1$  mm., on the manometer is noted, together with the temperature. The gas is then ejected from the chamber, the meniscus of the solution is lowered to the 2 ml. mark, and  $p_2$  is read on the manometer.

<sup>5</sup> If the 10 N NaOH used to neutralize the digest has been permitted to absorb a large amount of atmospheric  $CO_2$ , the gas extracted from the digest solution at this point will contain more  $CO_2$  than air, and the second extraction will yield a relatively large bubble, almost entirely  $CO_2$ . A large bubble on the second extraction does not indicate the necessity for a third extraction; the two extractions remove the air completely, and any  $CO_2$  that remains in the solution does not contaminate the  $N_2$  evolved by the hypobromite reaction, since the latter is carried out in strongly alkaline solution.

Blank determinations are performed with the entire procedure.

Calculations are made as described by Van Slyke (22) from the table of factors on p. 242 of his paper (22).

#### EXPERIMENTAL

##### *Preparation and Analysis of Total Protein Prepared from Plasma*

Total proteins were prepared from two different lots of Lyovac (lyophilized pooled normal human plasma of Sharp and Dohme) and from fresh dog plasma. Of the Lyovac, 2 gm. were dissolved in 50 ml. of water. Dog plasma was obtained from freshly drawn dog blood, with potassium oxalate as anticoagulant. Since the nitrogen values obtained are used to estimate the factor by which the nitrogen of normal human and dog plasma proteins is multiplied to calculate the protein, the purification of the proteins is outlined in some detail.

Lipides were removed by treatment with alcohol and ether. To 50 ml. of the Lyovac solution, or to 20 ml. of dog plasma to which 30 ml. of water had been added, 225 ml. of absolute alcohol, then 225 ml. of ethyl ether were added with shaking. After mixing well, the mixture was allowed to stand for 2 hours until the supernatant fluid was clear. About 300 ml. of the clear supernatant fluid were siphoned off. The precipitate, with the remainder of the supernatant, was transferred to a 250 ml. centrifuge bottle, centrifuged, and the supernatant fluid decanted off. The residue was washed once with 200 ml. of a mixture of 1 part of water to 4.5 parts each of absolute alcohol and ether. After thorough mixing with a footed rod, it was centrifuged and decanted. The residue was washed twice with 200 ml. portions of anhydrous ether in the same manner. The last traces of ether were removed from the residue in the centrifuge bottle by running a gentle current of air over the surface.

Mineral salts were removed by the procedure described by Robinson and Hogden (23). The finely powdered residue was completely dissolved in 200 ml. of water, the pH adjusted to 5.0 to 5.2 with 0.1 N acetic acid, and the solution placed in a boiling water bath for 1 hour. After cooling to room temperature, the coagulum was centrifuged, and the supernatant solution was removed by decantation. The residue was washed eight times with 150 ml. portions of boiling water, twice with 150 ml. portions of absolute alcohol, and three times with 150 ml. portions of anhydrous ether. The last traces of ether were removed from the residue by a gentle current of air.

The finely powdered residue was spread on hardened filter paper on a large watch-glass and air-dried by placing in a room with constant temperature of 23° and constant humidity of 30 per cent. After 40 hours the preparations were ground and well mixed and allowed to equilibrate for 24 hours longer, at which time they had come to constant weight.

Samples for all analyses were taken in closed vessels in the room in which the preparations had been air-dried. Carbon and hydrogen were determined by the micromethod of Pregl (13). Nitrogen was determined by the micro-Dumas procedure, by macro-Kjeldahl according to the procedure here described, and by the procedure of Campbell and Hanna (9), modified

by continuing the digestion for 2 hours, instead of 12 minutes, after the digest cleared.<sup>6</sup> Moisture content was determined by heating in an air oven at 102° to constant weight. Ash was determined on the residues from carbon determinations. Values for C, H, and N were corrected for moisture content of the air-dried preparations and for ash when present. Hydrogen was further corrected for the hydrogen of the water content. The results are shown in Table I.

The results obtained by the macro-Kjeldahl procedure with mercury as catalyst checked with those by the Dumas method within the limits of error

TABLE I  
*Elementary Analyses of Total Plasma Proteins Prepared from Dried Pooled Human Plasma and from Fresh Dog Plasma*

Analysis	Procedure used for analysis	Source of total plasma protein preparation			Average for human plasma
		Fresh dog plasma	Dried pooled human plasma, I	Dried pooled human plasma, II	
		<i>per cent of dry weight</i>	<i>per cent of dry weight</i>	<i>per cent of dry weight</i>	
Ash		0.14	0	0.31	
Moisture		11.78	9.84	9.37	
Carbon*	Combustion	53.25	53.57	53.25	53.41
Hydrogen†*	"	6.97	7.05	7.15	7.10
Nitrogen*	Dumas	16.38	15.96	16.08	16.02
"	Macro-Kjeldahl with Hg	16.33	16.07	16.04	16.06
"	Campbell and Hanna method with Se (9)	15.90	15.42		
Factor $\frac{\text{protein}}{\text{N}}$ for macro-Kjeldahl with Hg catalyst		6.12	6.22	6.24	6.23

\* Corrected for moisture and ash content.

† Corrected for hydrogen in H<sub>2</sub>O content of preparations.

of duplicate analyses. Macro-Kjeldahl analyses by the procedure of Campbell and Hanna (9) even when digestion was prolonged for 2 hours after clearing, gave only 97 per cent of the nitrogen determined by the Dumas method.

#### *Recovery of Ammonia by Macro Distillation in Presence of Mercury*

As controls for various procedures 10 ml. portions of 1 per cent solution of ammonium sulfate or 20 ml. of M/14 ammonium chloride solution were

<sup>6</sup> The digestion mixture of Campbell and Hanna is made by dissolving 10 gm. of CuSO<sub>4</sub>·5H<sub>2</sub>O and 10 gm. of selenium oxide in a mixture of 250 ml. of syrupy H<sub>3</sub>PO<sub>4</sub> and 750 ml. of concentrated H<sub>2</sub>SO<sub>4</sub>. Of this, 20 ml. are used for each macroanalysis.

diluted with water and distilled, after addition of 5 ml. of 18 N NaOH, into 50 ml. of 4 per cent boric acid, and titrated with N/14 sulfuric acid. The amounts of ammonia titrated in the distillates were taken as "100 per cent," for comparison with the amounts of ammonia obtained when mercury and other reagents were added. Distillations of ammonium sulfate or ammonium chloride were then performed in the presence of  $K_2SO_4$ ,  $H_2SO_4$ , and  $HgO$  in the amounts used in the macroprocedure described, with addition

TABLE II

*Recovery of Ammonia in Macro Distillation of Ammonium Salts in Presence of  $H_2SO_4$ ,  $K_2SO_4$ ,  $HgSO_4$ , and 18 N NaOH, with Various Procedures for Preventing Retention of Ammonia by  $HgO$*

Substance distilled	No. of determinations	Procedure for treating mercury	Ammonium distillate, mean yield
			<i>per cent</i>
$(NH_4)_2SO_4$	3	25 ml. 4% $K_2S$ added to acid mixture <i>before</i> NaOH	98.2
"	3	2.5 gm. solid $Na_2S_2O_3 \cdot 5H_2O$ added <i>after</i> NaOH	98.0
"	3	2.5 " " " " <i>before</i> "	99.7
"	2	5% $Na_2S_2O_3 \cdot 5H_2O$ dissolved <i>with</i> NaOH	99.4
$NH_4Cl$	7	2 gm. zinc dust added <i>before</i> NaOH	100.0

of potassium sulfide, sodium thiosulfate, or zinc dust to prevent retention of ammonia by the mercuric oxide. The results are shown in Table II.

#### *Effect of Digestion Rate and Time on Macro-Kjeldahl Procedure*

2 ml. samples of plasma were analyzed by the macro-Kjeldahl procedure described in this paper. When plasma was digested with rapid boiling, so that clearing occurred in 15 to 18 minutes, and boiling was continued for 0.5 and 1 hour after clearing, the results were 99.2 and 99.0 per cent of those by the procedure as described, in which "subboiling" was used throughout, and was continued for 2 hours after the digest became clear. With "subboiling" the preclearing period was 30 to 92 minutes. When the preclearing period required 50 to 92 minutes, maximum results were obtained even when the postclearing time was shortened to less than 2 hours. Digestion prolonged more than 2 hours after clearing gave no higher results than a 2 hour period. The blank determinations for the 8 hour digestion were twice as high as those for the 2 hour digestion. The results are shown in Table III.

TABLE III

*Analyses of 2 Ml. Samples of Plasma for Total Nitrogen by Present Macro-Kjeldahl Procedure, Varying Digestion Rate and Time*

Digestion before clearing		Digestion after clearing		Total digestion time	N found	Average N found	Per cent of digestion by subboiling with 30 min. to clear and 2 hrs. after clearing
Time	Conditions	Time	Conditions	hrs.	mg.	mg.	
min.		hrs.		hrs.	mg.	mg.	
15-18	Boiling	0.5	Boiling	0.75	21.07	21.14	99.2
					21.07		
					21.35		
15-18	"	1	"	1.25	21.29	21.11	99.0
					21.24		
					20.80		
50	Subboiling	0.5	Subboiling	1.25	21.43	21.37	100.2
					21.39		
					21.43		
92	"	0.5	"	2	21.21	21.26	99.7
					21.36		
					21.07		
92	"	1	"	2.5	21.15	21.39	100.3
					21.46		
					21.38		
30	"	1	"	1.5	21.40	21.08	98.9
					21.38		
					21.07		
30	"	2	"	2.5	21.02	21.32	100.0
					21.15		
					21.23		
30	"	4	"	4.5	21.33	21.22	99.5
					21.34		
					21.36		
30	"	8	"	8.5	21.31	21.31	100.0
					21.17		
					21.01		
					21.37		
					21.14		
					21.49		
					21.34		
					21.28		

*Effect of Selenium in Addition to Mercury As Catalyst in Macro-Kjeldahl Procedure*

Dog plasma was analyzed by the macroprocedure as described, with mercury as catalyst, and by the same procedure with addition of 0.15 gm. of selenium powder.



It appears from Table IV that selenium and mercury together give digestion not significantly more rapid or complete than mercury alone.

*Comparison of Methods for Determination of Total Nitrogen of Plasma by Various Macro-Kjeldahl Procedures*

An approximately 7 per cent solution of Lyovac (lyophilized pooled human plasma, Sharp and Dohme), two pooled dog plasmas, and a pooled human plasma were digested by the method described in this paper with mercury as catalyst, and by the procedure of Campbell and Hanna (9) with Se and Cu as catalysts. In distillation of the digests containing mercury, thiosulfate was used instead of zinc dust, as these analyses were done before

TABLE IV  
*Total Nitrogen of Plasma by Macro-Kjeldahl with Mercury As Catalyst with and without Addition of Selenium; Zinc Dust to Remove HgO during Distillation*

Source of plasma used (2 ml. samples)	Time digested after clearing	Catalysts used					
		Mercuric oxide			Mercuric oxide + selenium		
		Time required for digest to clear	N per 2 ml. plasma	N, per cent of 2 hr. digestion	Time required for digest to clear	N per 2 ml. plasma	N, per cent of 2 hr. digestion without Se
Dog	hrs.	min.	mg.		min.	mg.	
	0	14*	17.58	98.9	12*	17.55	98.7
	0	27†	17.54	98.6	25†	17.52	98.6
	0.5	†	17.73	99.7	†	17.73	99.7
Human	2.0	†	17.78	100.0	†	17.72	99.7
	0.5	40†	19.23	98.7	40†	19.36	99.3
	2.0	40†	19.49	100.0	40†	19.41	99.6

\* Boiling.

† Subboiling.

the use of zinc had been adopted. As shown in Table I, the ammonia yields from the digests with mercury might have been slightly higher if zinc had been used in the distillation. The results in Table V indicate that the digest mixture with mercury gave higher results than any of the other mixtures. The difference might have been still a little greater if zinc had been used in the distillation.

*Recovery of Ammonia from NH<sub>4</sub>Cl by Steam Distillation in Presence of All Reagents Used for Micro-Kjeldahl Procedure with Mercury As Catalyst*

Samples of standard NH<sub>4</sub>Cl solutions, containing respectively 1, 0.5, and 0.2 mg. of nitrogen, were measured into micro-Kjeldahl digestion tubes.

All reagents used for micro digestion were added and the contents of the tubes were distilled as described in the microprocedure. The ammonia was received into 10 ml. of 0.01428 N  $\text{H}_2\text{SO}_4$ . The volume of distillate collected was 50 ml.; this was the volume found adequate for quantitative distillation of ammonia in the still that was used. (The volume of distillate required

TABLE V

*Comparison of Methods for Determination of Total Nitrogen of Plasma by Various Macro-Kjeldahl Procedures*

Source of plasma used (2 ml. samples)	Time digested after clearing hrs.	Procedure used							
		Digest, 10 gm. $\text{K}_2\text{SO}_4$ , 1 gm. $\text{HgO}$ , 20 ml. $\text{H}_2\text{SO}_4$ . Distillation with sodium thiosulfate dissolved in				Digestion mixture of Campbell and Hanna (9) with Se and Cu as catalysts		10 gm. $\text{K}_2\text{SO}_4$ , 0.2 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 20 ml. $\text{H}_2\text{SO}_4$	10 gm. $\text{K}_2\text{SO}_4$ , 0.2 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 20 ml. $\text{H}_2\text{SO}_4$ , 0.5 ml. $\text{H}_2\text{O}$
		Added NaOH		Diluted acid digest, then NaOH added					
		N in sample analyzed	Per cent of 2 hr. digestion with $\text{HgO}$	N in sample analyzed	Per cent of 2 hr. digestion with $\text{HgO}$	N in sample analyzed	Per cent of 2 hr. digestion with $\text{HgO}$	N in sample analyzed	Per cent of 2 hr. digestion with $\text{HgO}$
Solution of Lyovac*	0.5			17.11	99.1	16.76	98.0		
	1			17.18	99.5				
	2			17.27	100.0	17.03	98.6		
	4					17.03	98.6		
Pooled dog Plasma 1	0.5					22.50	97.1		
	1					22.71	98.0		
	2	23.16	100.0			22.85	98.5		
Pooled dog Plasma 2	0.5	22.63	99.8						
	2	22.68	100.0			22.37	98.6		
	4					22.54	99.3		
	8					22.63	99.7		
Pooled human	2			23.03	100.0				
	4					22.65	98.4	22.13	96.1
								22.06	95.8

\* Lyophilized pooled human plasma, Sharp and Dohme.

was determined by distilling samples of  $\text{NH}_4\text{Cl}$  containing 4 mg. of nitrogen, collecting varying volumes of distillate, from 25 to 50 ml. 25 ml. of distillate showed 99.6 per cent of the theoretical value, and 50 ml. gave the theoretical values.)

The recovery of ammonia by distillation of samples of  $\text{NH}_4\text{Cl}$  containing 0.2 to 1 mg. of nitrogen varied from 99.6 to 100.4 per cent of the theoretical values, as shown in Table VI.

TABLE VI

*Recovery of Ammonia from  $\text{NH}_4\text{Cl}$  by Steam Distillation in Presence of All Reagents Used for Present Titrimetric Micro-Kjeldahl Procedure with Mercury As Catalyst*

Nitrogen in $\text{NH}_4\text{Cl}$ sample	
Theoretical	Recovered in distillate
mg.	per cent
1	100.0
1	99.8
1	99.8
0.5	100.4
0.5	100.0
0.5	99.6
0.2	100.0
0.2	100.0

TABLE VII

*Determination of Total Nitrogen of Plasma by Macro and by Micro Titrimetric Kjeldahl Procedures Described*

Procedure used	Plasma in sample analyzed	N found in sample analyzed		N found per 100 ml. plasma		N found in per cent of result by macro-procedure	
		Replicates	Mean	Replicates	Mean	Replicates	Mean
Macro	ml.	mg.	mg.	mg.	mg.		
	2	19.38		969.0			
	2	19.35		967.5			
	2	19.34		967.0			
	2	19.37	19.36	968.5	968.0		100.0
Micro	0.2	1.932		966.0		99.8	
	0.2	1.932		966.0		99.8	
	0.2	1.940	1.935	970.0	967.3	100.2	99.9
	0.1	0.972		972.0		100.4	
"	0.1	0.968		968.0		100.0	
	0.1	0.978		978.0		101.0	
	0.1	0.970	0.972	970.0	972.0	100.2	100.4
	0.05	0.484		968.0		100.0	
"	0.05	0.482		964.0		99.6	
	0.05	0.482		964.0		99.6	
	0.05	0.488	0.484	976.0	968.0	100.8	100.0
	0.02	0.198		990.0		102.3	
"	0.02	0.198		990.0		102.3	
	0.02	0.196	0.197	980.0	986.7	101.2	101.9

*Determination of Total Nitrogen of Plasma by Present Macro and Micro Titrimetric Kjeldahl Procedures*

The plasma used for the analyses was an approximately 7 per cent solution of Lyovac (a lyophilized pooled human plasma prepared by Sharp and Dohme). For macro-Kjeldahl analyses samples of 2 ml. were taken. For micro-Kjeldahl analyses 10- and 50-fold dilutions of the plasma were prepared with 0.85 per cent sodium chloride solution. Samples as designated in Table VII were taken for analyses, covering a range of approximately 0.2 to 2 mg. of nitrogen. The results are given in Table VII.

TABLE VIII

*Total Nitrogen Determined in Samples of 0.1 Ml. of Pooled Plasma by Present Gasometric Micro-Kjeldahl Procedure*

$P_{N_2}$  mm.	Temperature  °C.	Factor	Nitrogen in sample	
			Found  mg.	Per cent of N by macromethod*
377.8	23.0	0.003156	1.192	100.1
377.4	23.3	53	1.190	99.9
377.8	23.5	50	1.190	99.9
378.0	24.0	45	1.189	99.8
377.4	24.1	44	1.187	99.7
378.5	24.4	41	1.189	99.8
379.0	24.5	40	1.190	99.9
378.8	24.6	39	1.189	99.8
Mean.....			1.190	99.9

\* Nitrogen by macro-Kjeldahl was 11.91 mg. per ml.

*Comparison of Total Nitrogen of Plasma Determined by Present Macro Titrimetric and Micro Gasometric Procedures*

2 ml. samples of pooled human plasma were analyzed by the macro-Kjeldahl procedure. The mean result was taken as 100 per cent for comparison with results by the microprocedure. For the microanalyses the plasma was diluted 10-fold, and 1 ml. samples of the dilute solution were taken for analysis. The results are given in Table VIII.

### DISCUSSION

*Nitrogen Content of Plasma Proteins*—Total plasma proteins prepared from two pools of human plasma, analyzed for total nitrogen by the macro-Kjeldahl procedure described, with mercury as catalyst, gave a mean value

of 16.06 per cent, which checked with the mean value of 16.02 per cent by the Dumas method. The macro-Kjeldahl procedure of Campbell and Hanna (9) with digestion for 2 hours after clearing and with Se and Cu as catalysts gave a value of 15.42 per cent.

The literature on the analysis of plasma proteins reports varying nitrogen percentages. The lack of agreement is in part probably due to absence of exact data on the moisture and ash contents of the proteins analyzed. Proteins are so hygroscopic that completely dried samples cannot be weighed with accuracy. The most uniform analyses are obtained, according to the writers' experience, when the protein is air-dried by equilibration with the moisture of the atmosphere, and samples are weighed for all analyses, including moisture and ash, during a short time interval and under the atmospheric humidity and temperature conditions of equilibration. In part it appears that the inconstant and usually low nitrogen values in the literature may have been due to the use of inadequate digestion procedures for Kjeldahl analyses.

Bierry and Vivario (24) reported a mean nitrogen value of 15.26 per cent for preparations of total human plasma proteins. They did not give ash or moisture content, and did not indicate the procedure for the nitrogen analyses. Block and his associates (25-27) reported numerous analyses of total proteins of human serum, giving nitrogen values varying from 14.3 to 15.3 per cent. Analyses were performed by macro-Kjeldahl, but the procedure used was not stated. Some of the samples were dried at 110° (25, 27); other results (26) are given without mention of drying or of ash or moisture content. Murrill, Block, and Newburgh (28) prepared proteins from a pool of three human sera, but did not determine ash or moisture content. Nitrogen determined by the micro-Kjeldahl procedure of Pregl was 14.28 per cent. Robinson and Hogden (23) prepared proteins from three human sera. Dried ash-free samples were weighed. Nitrogen determined by macro-Kjeldahl, with a digestion mixture of  $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$ , and  $\text{SeOCl}_2$ , gave values between 15.5 and 16.0 per cent, mean 15.72. Cook (29) prepared proteins from four pools of human plasma. Dried samples were weighed. Nitrogen determined by the micro-Kjeldahl procedure of Chibnall, Rees, and Williams (30), with  $\text{H}_2\text{SO}_4$ ,  $\text{K}_2\text{SO}_4$ ,  $\text{CuSO}_4$ , and  $\text{Na}_2\text{SeO}_4$ , gave values between 14.71 and 15.54 per cent, mean 15.23. Nitrogen determined by these authors by micro-Dumas gave values between 13.29 and 15.96 per cent, mean 15.20. No mention is made of ash content. They concluded that the proper factor for conversion of nitrogen to total plasma protein was 6.6. Brand, Kassell, and Saidel (31) and Brand (32) analyzed fractions of human plasma proteins for nitrogen by the micro-Dumas method, in which they used air-dried samples and corrected for ash and mois-

ture content. In the fractions which were pure (albumin and  $\gamma$ -globulin) they found 15.95 per cent nitrogen for albumin and 16.03 for  $\gamma$ -globulin. The factors for conversion of nitrogen to protein calculated from these values are 6.27 for albumin and 6.24 for  $\gamma$ -globulin, agreeing closely with the factor found in the present paper for total proteins of normal human plasma.

The factor 6.25, for conversion of nitrogen to protein, has been generally used for at least 75 years. Its origin is obscure and difficult to trace. It has long been known that different proteins vary considerably in their nitrogen content, and that for each protein its own factor must be established before exact calculation of the weight of dry protein present can be made from nitrogen determinations. From the data given in this paper for total human plasma proteins, and the data of Brand and his associates for pure human plasma albumin and  $\gamma$ -globulin, the factor 6.25 seems justifiable, for the present, for the calculation of weights of plasma proteins from nitrogen.

Some of the markedly higher values for the factor for plasma proteins, derived from other analyses in the literature, appear to be attributable to low nitrogen values by the Kjeldahl procedures used. For our own protein preparations the factor calculated from the determination of nitrogen by the procedure of Campbell and Hanna (9) is 6.49, because the procedure gave nitrogen values 3 per cent lower than the Dumas combustion or Kjeldahl digestion with mercury as catalyst.

*Losses with Selenium and Mercury As Catalysts*—The literature indicates that when selenium is used as a catalyst for the Kjeldahl procedure the length of time for digestion should be carefully established for the substance analyzed. Sandstedt (33) reported that the loss of nitrogen on long heating with selenium is greater than with other catalysts. Davis and Wise (34) and Illarionov and Ssolovjeva (35) reported a loss of nitrogen if heating was longer than 35 minutes with selenium. Dalrymple and King (36) found that digestion for 1 hour with  $\text{HgO}$  and  $\text{K}_2\text{SO}_4$  gave the same result as digestion for 3 to 6 hours with selenium or 1.5 hours with various selenates. With selenates the recovery of nitrogen rose with time of digestion to a maximum, then diminished with progressive loss of nitrogen. Patel and Sreenivasan (37) reported progressive losses of nitrogen during digestion of casein with selenium or selenium plus mercuric oxide as catalysts if digestion was prolonged beyond 15 minutes after clearing. Osborn and Krasnitz (38) reported that when the digestion period was extended danger of loss of nitrogen with different catalysts increased in the order of  $\text{Hg}$ ,  $\text{Se}$ ,  $\text{Se} + \text{Hg}$ . Use of mercury involved the least danger of loss.

Determinations of carbon, hydrogen, and of nitrogen by the Dumas method were performed by Dr. A. Elek, to whom we wish to express our gratitude.

## SUMMARY

Only digestion mixtures containing mercury as catalyst have been found to give nitrogen values for proteins as high as the values yielded by Dumas combustion.

Methods are described in which digestion mixtures yielding nitrogen values equal to those of the Dumas combustion are employed, and in which the ammonia formed is determined by macro or micro titration, or by micro gasometric measurement.

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# DETERMINATION OF PROTEIN IN URINE BY THE BIURET METHOD

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(Received for publication, July 15, 1948)

A method for the determination of protein in urine by means of the biuret reaction was described by Hiller (1) and by Hiller, McIntosh, and Van Slyke (2), in which pure biuret prepared by Kahlbaum was used as a standard for visual colorimetry. Since Hiller (1) the biuret method has been used for the determination of urinary proteins by Price (3) and by Lehmann (4), and has been widely used in many forms and modifications for the determination of plasma proteins. A review of the literature on the biuret method will not be given here, but may be found in the papers of Küntzel and Dröschner (5) and of Robinson and Hogden (6). The latter workers studied the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration and also published transmission curves for the biuret color.

The present paper describes a photometric method for the determination of urinary protein in which the entire procedure is carried out in a single test-tube, which serves as a cuvette for the photoelectric spectrophotometer. The method can be used even when the urine contains Evans blue excreted after blood volume determinations. Since pure preparations of biuret are not now obtainable on the market, solutions of urinary proteins, of concentration determined by accurate Kjeldahl analysis, are used to prepare standard optical density curves, and the use of a chrome-alum solution for checking the curves is detailed.

## METHOD

### *Apparatus*

*Cuvettes*, 15 by 100 mm. (outer measurements). Test-tubes, thick wall, without lip, from the Arthur H. Thomas Company, No. 9446, make good inexpensive cuvettes. After cleaning with dichromate cleaning mixture the tubes are tested for uniformity by reading the optical density of a solution containing approximately 15 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per 100 ml. in each tube in a spectrophotometer at a wave-length of  $560\text{ m}\mu$ . Tubes which read within an optical density variation of  $\pm 0.0015$  of the average are chosen as cuvettes. These are then etched with numbers and calibrated

to contain 10 ml. The tubes must be carefully handled to prevent scratching. Before each series of readings in the spectrophotometer, finger-marks must be removed by wiping the outside of the tube first with a damp, then with a dry towel. They must be cleaned immediately after use. A good cleaning procedure is to remove precipitates by rinsing and shaking with water; then small amounts of copper sulfate which adhere to the upper part of the inner walls of the tubes are removed with dilute hydrochloric acid, approximately 1 N. The tubes are then rinsed five times with tap water and three or four times with distilled water and inverted in a test-tube rack to drain onto a towel. Only the tubes used for blank determinations need be dry. The tubes used for protein analysis can be used without drying.

*Rubber stoppers*, solid No. 0, to fit cuvettes.

*Centrifuge metal shields*  $3\frac{1}{8}$  inches long and  $\frac{3}{4}$  inch in diameter will fit the size of cuvettes used. With an eight place head and three or four place trunnion carriers a large number of tubes can be centrifuged at one time.

*Photoelectric spectrophotometer*. The Coleman junior model was used, but any type can be used, with a cuvette holder 3 inches in length. If the holder is too wide for the cuvettes, a ring of hard rubber can be fitted into the top of the holder to keep the cuvettes in a vertical position.

A 5 ml. burette marked at 0.25 ml. intervals to deliver the copper sulfate solution.

A 25 ml. burette to deliver trichloroacetic acid.

*Dispenser for sodium hydroxide*. An aspirator bottle or a separatory funnel type of vessel, equipped with a soda lime tube and an outlet tube with a small opening for delivery of small drops.

### Reagents

*Trichloroacetic acid*, 10 per cent solution. Keep in the refrigerator when not in use.

*Copper sulfate*, 20 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per 100 ml.

*Sodium hydroxide*, reagent grade, 3 per cent solution. This solution is stored in the dispenser described under "Apparatus," and should not be allowed to age beyond 2 weeks, as the solution on standing over a longer period of time in glass gives higher blanks and lower protein values than when freshly made (see Table III).

### Procedure

If the urine contains a precipitate, it should be filtered before analysis.

*Precipitation of Protein*—Enough urine to contain between 5 and 20 mg. of protein is pipetted into the bottom of each cuvette. The samples of albuminous urine usually range from 0.5 to 5 ml. to contain the desired amount of protein. If the concentration of protein is higher than 30 mg.

per liter, the urine is diluted with water so that a sample will contain between 5 and 20 mg. of protein. To the urine in each cuvette an equal volume of 10 per cent trichloroacetic acid is added. If the total volume of urine plus trichloroacetic acid is 6 ml. or less, mixing can be accomplished by shaking the cuvette. If the volume is greater than 6 ml., a thin stirring rod should be used to get complete mixture, with care not to scratch the wall of the cuvette. The rod is washed down with a few drops of water. The cuvette is allowed to stand 10 minutes, then centrifuged at about 2500 R.P.M. for 10 minutes. The supernatant solution is decanted and the cuvette is inverted against a towel or filter paper for a moment to drain.

*Dissolving Protein Precipitate*—The protein precipitate is dissolved by adding 2 ml. of 3 per cent sodium hydroxide. The cuvettes are shaken occasionally until solution is complete, but shaking must be gentle to avoid formation of foam. If cuvettes containing appreciable amounts of protein are allowed to stand for a time without occasional shaking, undissolved protein at the bottom of the cuvette will form a clear gel and may be overlooked, as it is difficult to see and requires a longer time to dissolve than does the white precipitate. If the cuvette is examined with a window or light as background, while whirling gently, a spiral of dissolving protein can be seen rising from the bottom of the cuvette. Solution is complete when there are no transparent strands on shaking the solution. More 3 per cent sodium hydroxide solution is then added with occasional shaking, until the volume in the cuvette reaches the 10 ml. mark.

Two dry cuvettes are filled to the 10 ml. mark with 3 per cent sodium hydroxide to serve as blanks.

*Setting Zero Point of Photometer*—The zero optical density point is set before each reading with the holder in place, but without a cuvette (air zero).

*Blanks*—The density of the 3 per cent NaOH in the cuvettes is read as  $D_{B_1}$ , Blank 1.

*Reading of Optical Density,  $D_1$ , Due to Pigments in Urine Carried Down by Precipitate of Protein*—The tubes containing redissolved protein are wiped and the optical density is read as  $D_1$  at wave-length 560 m $\mu$ .

*Development of Biuret Color and Reading of Optical Density,  $D_2$* —After  $D_1$  readings have been made, 0.25 ml. of 20 per cent copper sulfate is added from the 5 ml. marked burette to each protein solution and blank. Each tube is stoppered as soon as the copper sulfate has been added, and is immediately shaken vigorously about 15 times. Unless shaking is prompt, clumps of copper hydroxide may stick to the sides. Stoppers are removed and washed. The tubes are allowed to stand 10 minutes for the biuret color to develop, and are then centrifuged for 4 minutes at about 2500 R.P.M. (Blanks should not stand longer than 10 minutes before centrifuga-

tion as they tend to be lowered on standing.) They are then wiped clean and the optical density is read at 560  $m\mu$ , with the zero point set as described above. The readings of the protein solutions are recorded as  $D_1$  and the blanks are  $D_{B_2}$ , Blank 2. Tubes should be washed immediately after use as described under "Apparatus."

### Calculation

$D_1$  = optical density reading of 10 ml. of protein solution  
 $D_{B_1}$  = " " " " solvent (3 per cent NaOH) = Blank 1  
 $D_2$  = " " " " 10.25 ml. of protein solution + copper sulfate  
 $D_{B_2}$  = optical density reading of reagent blank (NaOH +  $CuSO_4$ ) = Blank 2  
 $D_P$  = " " " due to biuret color formed from protein in a volume of 10 ml.

$$(1) \quad D_P = 1.025 (D_2 - D_{B_2}) - (D_1 - D_{B_1})$$

The mg. of protein in the sample analyzed are read from a curve constructed for this purpose, relating  $D_P$  to mg. of protein for the specific set of cuvettes and spectrophotometer used.

$$\text{Mg. protein per liter urine} = \frac{\text{mg. protein in sample analyzed} \times 1000}{\text{ml. urine in sample taken}}$$

*Construction of Curve Relating Optical Density to Mg. of Protein in Sample Analyzed*—The relationship between optical density reading and protein concentration depends on the specific set of cuvettes and on the spectrophotometer used. It is therefore imperative that a curve be constructed for each set of apparatus.

Several urine samples containing protein can be used for this purpose. The urines are analyzed for total nitrogen and non-protein nitrogen by the macro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (7), who found that the best results of analyses of plasma protein were obtained when mercury was used as catalyst. For determination of non-protein nitrogen the protein is precipitated under the same conditions as for the biuret method; equal volumes of urine and 10 per cent trichloroacetic acid are mixed, let stand 10 minutes or longer, then centrifuged. Aliquot portions of the supernatant solution are taken for analysis.

The calculation for the protein content of the urines is

$$\text{Protein per 100 ml.} = 6.25 [(\text{total N per 100 ml.}) - (\text{non-protein N per 100 ml.})]$$

Of each urine analyzed three or four samples of various size are chosen, containing amounts of protein ranging from about 3 to 15 mg. These are analyzed in triplicate by the biuret method, and the  $D_P$  values calculated by Equation 1 are plotted against the mg. of protein in the samples taken for analysis. A straight line curve can thus be constructed for calculations.

Such a curve was checked at frequent intervals and was found to remain constant over a period of 6 months.

### EXPERIMENTAL

*Choice of Wave-Length for Reading Biuret Color*—Optical densities of the biuret color prepared from a sample of urine protein were read at various wave-lengths in the Coleman junior photoelectric spectrophotometer. A curve constructed from these data showed a maximum optical density at wave-lengths between 550 and 570  $m\mu$ , and was almost identical with the curve of Robinson and Hogden (6). A wave-length of 560  $m\mu$  was chosen.

*Reproducibility of Results by Biuret Method*—Urine samples were chosen which contained varying amounts of protein, between 3 and 19 mg.

TABLE I  
*Reproducibility of Results by Biuret Method for Various Amounts of Protein in Sample of Urine Analyzed*

Urine sample ml.	Protein found in sample; mean of 20 determinations mg.	Standard deviation from mean	
		mg.	per cent of mean
0.5	3.105	$\pm 0.065$	$\pm 2.09$
1	6.328	$\pm 0.077$	$\pm 1.22$
2	12.798	$\pm 0.126$	$\pm 0.99$
3	19.030	$\pm 0.171$	$\pm 0.90$

Twenty analyses were performed on each sample chosen. The results are shown in Table I.

*Stability of Biuret Color*—On six urine specimens which were analyzed by the biuret method, optical density readings were taken within  $\frac{1}{2}$  hour after development of the biuret color. The cuvettes were stoppered and let stand at room temperature, 22°, in daylight but not in direct sunlight, and readings were repeated at 3 and 4 hours after color development. The results, recorded in Table II, show no appreciable change in optical density over a period of 4 hours.

*Effect of Age of 3 Per Cent Sodium Hydroxide Solution on Results Obtained by Biuret Method*—3 per cent sodium hydroxide solutions were protected from atmospheric CO<sub>2</sub> and were used after standing in glass containers for intervals up to 90 days, as indicated in Table III. For the analyses a solution of serum albumin containing 3.9 mg. of protein per ml. was used. Samples of 1 ml. were analyzed in triplicate for each sodium hydroxide solution. All the analyses in Table III were done on the same day. Reagent blanks were determined for each solution used. The sodium hydrox-

ide solutions which were kept for 42 to 90 days all gave higher reagent blanks. The results shown in Table III indicate that the 3 per cent so-

TABLE II

*Stability of Biuret Color on Standing at Room Temperature in Daylight but Not in Sunlight; Optical Densities with Air As Zero*

Urine No.	Optical density readings; time after biuret color development		
	Within $\frac{1}{2}$ hr.	After 3 hrs.	After 4 hrs.
Water	0.050	0.050	0.050
Reagent blanks	0.081	0.080	0.080
	0.081	0.080	0.080
152	0.441	0.440	0.440
153	0.391	0.391	0.391
154	0.233	0.233	0.234
155	0.349	0.349	0.350
156	0.300	0.300	0.300
164	0.590	0.590	0.590

TABLE III

*Effect of Age of 3 Per Cent Sodium Hydroxide Solution on Biuret Results*

Age of NaOH	Protein found in sample	Deviation from protein found with freshly made NaOH
<i>days</i>	<i>mg.</i>	<i>mg.</i>
0	3.9	
1	3.8	-0.1
2	3.9	0
3	3.9	0
4	3.8	-0.1
5	4.0	+0.1
6	3.7	-0.2
7	4.0	+0.1
13	3.8	-0.1
17	3.7	-0.2
19	3.7	-0.2
42	3.6	-0.3
49	3.7	-0.2
60	3.6	-0.3
90	3.7	-0.2

dium hydroxide solution may be used for a period of 2 weeks without appreciably affecting the accuracy of the procedure.

*Correction for Urinary Pigments in Biuret Method*—When protein is precipitated from a highly concentrated urine, some of the pigment is carried down with the precipitate. When such a precipitate is dissolved in alkali,

the solution is sufficiently colored to give a slight optical density reading at the wave-length at which the biuret color is read.

To determine the amount of error introduced into the biuret method by this color, a serum albumin solution containing 10.6 mg. of protein per ml. was analyzed before and after addition of a dilute normal, straw-colored urine and of a concentrated normal, deeply colored urine. The results were calculated with and without the correction for the color of the protein solution (optical density readings  $D_1$  in the method). Column 4 of Table IV shows that with varying amounts of protein in the sample the corrected readings indicate, within the limits of error of the method, the amount of protein present. When the calculations are made without correcting for pigment (Columns 5 and 6, Table IV), the results are increased to a degree

TABLE IV  
*Effect of Urinary Pigments on Biuret Method*

Normal urine		Serum albumin solution	Protein in sample calculated from added albumin	Protein in sample by biuret method		Protein uncorrected, in per cent of corrected
Color	Sample taken (1)			Corrected for pigment (4)	Uncorrected for pigment (5)	
	ml.	ml.	mg.	mg.	mg.	(6)
	0	1	10.6	10.6	10.6	100.0
Dark	3	0.25	2.65	2.7	3.0	110.1
"	3	0.5	5.3	5.5	5.8	105.5
"	3	1	10.6	10.6	10.9	103.8
"	3	1.5	15.9	15.8	16.3	103.1
	4	1	10.6	10.7	11.2	103.6
Light	4	1	10.6	10.6	10.9	102.8

beyond the experimental error of the method. This error becomes relatively greater when smaller amounts of protein are analyzed.

The dye, Evans blue (T-1824), when injected into patients with proteinuria for the determination of plasma volume, is excreted bound to the urinary protein. A number of urines containing T-1824 were analyzed for protein by macro-Kjeldahl determination and by the biuret method. Results by the biuret method were calculated with and without the correction for dye (included in the  $D_1$  readings). Table V shows that results calculated with the correction check with the results by Kjeldahl analysis within the limits of error, whereas the uncorrected photometric results are higher.

*Comparison of Determinations of Urine Protein by Macro-Kjeldahl, by Biuret Method, and by Sedimentation Method of Shevky and Stafford (8) As Modified by MacKay (9)*—Twelve urines were analyzed for protein (1) by

the macro-Kjeldahl procedure described for construction of the curve for conversion of optical density to protein content, (2) by the biuret method, and (3) by the sedimentation method of Shevky and Stafford (8) as modi-

TABLE V  
*Analyses of Urine Containing Evans Blue (T-1824) by Biuret Method*

Urine specimen	Protein per liter			Biuret, per cent of Kjeldahl	
	Macro-Kjeldahl	Biuret method		Corrected for dye	Uncorrected for dye
		Corrected for dye	Uncorrected for dye		
	gm.	gm.	gm.		
B-251	13.8	14.0	14.5	101.5	105.0
B-147	33.0	33.0	33.5	100.0	101.5
Se	10.5	10.4	10.8	99.1	102.9
C	17.5	18.0	18.2	102.8	104.0
M-173	28.2	28.0	28.6	99.1	101.5
B-171	41.5	41.0	42.0	98.8	101.2
M-177	20.7	21.0	21.4	101.5	103.4

TABLE VI  
*Comparison of Determinations of Urine Proteins by Macro-Kjeldahl, by Biuret Method, and by Method of Shevky and Stafford*

Urine specimen	Protein per liter			Per cent deviation from Kjeldahl	
	Macro-Kjeldahl	Biuret method	Shevky-Stafford	Biuret method	Shevky-Stafford
	gm.	gm.	gm.		
B-147	33.0	33.0	47.2	0	+43.0
B-251	13.8	14.0	18.7	+1.5	+35.5
B-171	41.5	41.0	63.0	-1.2	+51.8
M-173	28.2	28.0	34.5	-0.9	+22.4
N-177	20.7	21.0	28.8	+1.5	+39.0
R	8.0	8.0	10.4	0	+30.0
W	6.3	6.3	7.6	0	+20.5
O'F	25.6	25.2	24.7	-1.5	-3.5
Sp	3.6	3.6	3.8	0	+5.5
Ro	4.5	4.5	5.1	0	+13.3
C	17.5	18.0	18.2	+2.8	+4.0
Se	10.5	10.4	10.8	-0.9	+2.9

fied by MacKay (9). In Table VI the results by the biuret method are seen to check with those by macro-Kjeldahl analysis within the limits of error. The results by the sedimentation method show deviations from the Kjeldahl, ranging from -3.5 to +51.8 per cent.

*Procedures for Frequent Checking of Calculation Curve—To find a pro-*



cedure for checking the calculation curve at frequent intervals a search was made for compounds which either give a biuret reaction or which give an optical density curve similar to that of the biuret color.

Chromic ammonium sulfate,  $\text{Cr}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ , was chosen because in aqueous solution its transmission curve is near that of the biuret color, with a minimum transmission between wave-lengths of 570 and 590  $\text{m}\mu$ . Aqueous solutions of 0.5 and 0.25 per cent were prepared. These solutions showed a color instability when first made up. A daily increase was found in the optical density readings until the 4th day, but thereafter the readings remained unchanged when made at intervals up to 1 year. The optical density readings of the two solutions were located on the calculation curve and checked at intervals. Two preparations of chromic ammonium sulfate, one Baker's "analyzed," one a c.p. product obtained from the Fisher Scientific Company, gave the same results.

#### SUMMARY

A biuret method for the determination of urinary protein is described in which the entire procedure is carried out in a single test-tube which serves as a cuvette for the photoelectric spectrophotometer. Results agree with those by an accurate macro-Kjeldahl method (digestion with mercury catalyst), the standard deviation from the Kjeldahl values being of the order of  $\pm 1$  per cent when the urine samples contained 6 to 19 mg. of protein.

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# SUBSTITUTES FOR SAPONIN IN THE DETERMINATION OF OXYGEN AND CARBON MONOXIDE OF BLOOD

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(Received for publication, July 15, 1948)

When oxygen or carbon monoxide in blood is determined by setting these gases free from  $\text{HbO}_2$  or  $\text{HbCO}$  by means of ferricyanide, it is necessary to lake the cells completely, because ferricyanide does not penetrate the intact erythrocytes. The hemolytic agent used by Van Slyke and Neill (1) to lake the cells was saponin.

During the past few years commercial preparations of saponin which have been used as a hemolytic agent in the determination of oxygen in blood by the method of Van Slyke and Neill (1) have been found in this laboratory to give results considerably lower than those obtained with a Kahlbaum preparation of saponin which had been previously in use.

The use of Duponol WA and Duponol W-20 as a substitute for saponin in the determination of oxygen was reported by Vestling and Swerdlow (2). They used the method of Sendroy (3) for the determination of oxygen capacity, in which 32 per cent potassium ferricyanide containing the hemolytic agent is added to the blood after saturation of the blood with air (oxygen) in the chamber of the blood gas apparatus of Van Slyke and Neill (1). Ramsay (4) used oleyl sodium sulfate as a substitute for saponin, and warned that an excess causes stop-cocks to leak and impairs greatly the hemolytic action.

The presence and distribution of saponins in plants and their value as hemolytic agents have been investigated by Luft (5).

The present paper describes procedures in which urea or infusions of senega root, root soapwort, and quillaja soap bark, which are commercially available,<sup>1</sup> can be used as hemolytic agents in the determination of oxygen in blood.

Evidence is given that hemoglobin can be determined by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz (6) without addition of a hemolytic agent such as saponin.

## METHODS

### *Determination of Oxygen in Blood with Urea As Hemolytic Agent*

The method of Van Slyke and Neill (1) is used with slight modifications.

<sup>1</sup> Senega root, N. F. (cut and sifted), root soapwort (cut), and quillaja soap bark (cut) were obtained from the Amend Drug and Chemical Company, Inc., 117-119 East 24th Street, New York 10.

*Reagents*

*Urea*, 40 per cent solution, containing 1 per cent egg albumin.

*Potassium ferricyanide*, 32 per cent solution.

*Sodium hydroxide*, 1 N air-free solution.

*Sodium hyposulfite*, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, air-free solution, described by Van Slyke (7).

*Caprylic alcohol*.

*Procedure for 1 Ml. Samples of Blood*

2 drops of caprylic alcohol are drawn into the capillary of the blood gas apparatus (1, 7), and 7.5 ml. of the urea solution are measured into the chamber. After making a mercury seal, the solution is deaerated by evacuating and shaking for 3 minutes. 6 ml. of the solution are run up into the cup; then 1 ml. of blood is measured into the chamber, followed by 1 ml. of the solution in the cup. After making a mercury seal, the mixture is evacuated and shaken 3 minutes to take the blood. The vacuum is released, the upper cock being kept closed. In the cup of the gas apparatus place a few ml. of water and a little mercury. From a small rubber-tipped burette containing the 32 per cent potassium ferricyanide discard a drop so that there is no air space in the tip, insert the tip through the water and mercury into the bottom of the cup, and admit 0.2 ml. of the ferricyanide directly into the chamber. Seal with mercury, evacuate, and shake 3 minutes.

Carbon dioxide and oxygen are absorbed as usual (1), and pressure readings are taken with the meniscus at the 0.5 ml. mark.

Blanks are carried out by the procedure for blood, except that after deaeration of the urea solution 4 ml. are run up into the cup in place of 6 ml., and the blood sample is omitted. The ferricyanide is thus added to 3.5 ml. of deaerated urea solution in the chamber, instead of to a mixture of 2.5 ml. of that solution with 1 ml. of blood.

*Determination of Oxygen in Blood with Senega Root, Root Soapwort, or Quillaja Soap Bark As Hemolytic Agents**Reagents*

*Sodium hydroxide*, *sodium hyposulfite*, and *caprylic alcohol* are the same as described above.

*1.6 per cent potassium ferricyanide* in 0.5 per cent infusion of either senega root, root soapwort, or quillaja soap bark. The infusions are made by grinding the root or bark in a mortar or fine grinder and measuring 0.5 gm. into 100 ml. of water. The water is heated to boiling and the infusion is strained through finely woven cheese-cloth in a funnel.

### Procedure

The procedure is that described by Van Slyke and Neill (1), except that the ferricyanide-saponin solution of these authors is replaced by a solution of ferricyanide in one of the above infusions.

### EXPERIMENTAL

*Determination of Oxygen in Blood*—As controls by which to evaluate various procedures for the determination of oxygen in blood, blood was analyzed for oxygen capacity by the method of Van Slyke and Neill (1), except that the saponin-ferricyanide reagent contained 1.6 per cent potassium ferricyanide in place of 0.3 per cent. The saponin used was a Kahlbaum preparation which had long been in use in the laboratory and was known to give good results. The results by this procedure are recorded as 100 per cent in Table I.

A preparation of Baker saponin gave results which were 53.9 per cent of those with the Kahlbaum saponin when both were used in 0.3 per cent concentration in the ferricyanide solution. A preparation of saponin from the Amend Drug and Chemical Company, used in the same concentration, yielded 83.6 per cent. A concentration of 1 per cent yielded 98.8 per cent.

When water was substituted for the urea solution in the procedure described under "Methods," in which urea was used as hemolytic agent and the ferricyanide was added *after* laking of the blood, the yield was 97.3 per cent.

When 40 per cent urea was substituted for saponin, and the procedure was the same as for the controls above, the yield was 81.3 per cent and the blanks were high. When 1 per cent egg albumin was added in addition to urea, the yield was 92.8 per cent.

By the use of urea and egg albumin as described under "Methods," in which the blood is laked before addition of potassium ferricyanide, the yield was 99.5 per cent. The same procedure with omission of urea yielded 94.2 per cent.

Substitution of 0.5 per cent infusions of senega root, root soapwort, or quillaja soap bark for saponin gave results comparable to those with the Kahlbaum saponin. The results are shown in Table I.

*Simultaneous Determination of Carbon Dioxide and Oxygen in Blood*—The method of Van Slyke and Neill (1) was used, with the acid saponin-ferricyanide reagent described by Peters and Van Slyke (8). Two solutions are made: (a) 0.8 gm. of Kahlbaum saponin and 3.2 gm. of potassium ferricyanide diluted with water to 100 ml.; (b) 1 ml. of concentrated lactic acid of specific gravity 1.20 diluted to 100 ml. Before use equal volumes of the two solutions are mixed. Results with this reagent were used as controls by which to evaluate substitutes for saponin.

TABLE I

*Effects of Replacing Kahlbaum Saponin by Other Hemolytic Agents in Determination of Oxygen Capacity of Blood*

Blood No.	Hemolytic agent used	Procedure and reagents	Oxygen capacity		Mean in percent of result with Kahlbaum saponin
			Duplicates	Mean	
			vol. percent	vol. percent	
1	Kahlbaum saponin	Van Slyke-Neill procedure with 0.3% saponin in 1.6% potassium ferrieyanide as reagent	20.58 20.60	20.59	100.0
1	Baker saponin	" "	11.04 11.11	11.09	53.9
1	Amend saponin	" "	17.15 17.25	17.20	83.6
1	" "	Van Slyke-Neill with 1% saponin in 1.6% ferrieyanide	20.26 20.31	20.30	93.8
1	Water	Water deaerated, blood added, shaken 3 min. to hemolyze, then 0.2 ml. 32% ferrieyanide added	20.06 19.98	20.02	97.3
1	Urea	Van Slyke-Neill with 10% urea in 1.6% ferrieyanide as reagent	16.72 16.75	16.74	81.3
1	Urea + egg albumin	Van Slyke-Neill with reagent containing 10% urea, 1% egg albumin, 1.6% ferrieyanide	19.12 19.05	19.09	92.8
2	Kahlbaum saponin	Van Slyke-Neill with 0.3% saponin in 1.6% ferrieyanide as reagent	18.98 18.98	18.98	100.0
2	Urea + egg albumin	10% urea in 1% egg albumin, deaerated, blood added, shaken 3 min. to hemolyze, then 0.2 ml. 32% ferrieyanide added	18.86 18.92	18.89	99.5
2	Egg albumin	As above, urea omitted	17.92 17.85	17.89	94.2
2	Senega root	Van Slyke-Neill with 1.6% ferrieyanide in 10% infusion of senega root as reagent	18.94 18.97	18.96	99.9
3	Kahlbaum saponin	Van Slyke-Neill with 0.3% saponin in 1.6% ferrieyanide	22.89 22.90	22.90	100.0
3	Senega root	Van Slyke-Neill with 1.6% ferrieyanide in 0.5% infusion of senega root as reagent	22.89 22.98	22.94	100.2
3	Root soapwort	Van Slyke-Neill with 1.6% ferrieyanide in 0.5% infusion of root soapwort as reagent	22.82 22.80	22.81	99.6
3	Quillaja soap bark	Van Slyke-Neill with 1.6% ferrieyanide in 0.5% infusion of quillaja soap bark as reagent	22.97 22.83	22.90	100.0

As substitutes for saponin, senega root and root soapwort were used. For these substitutes solution (a) was prepared by dissolving 3.2 gm. of

potassium ferrieyanide in 100 ml. of 2 per cent infusion of the root. The infusions were prepared as described under "Methods."

TABLE II

*Effect of Using Substitutes for Saponin in Simultaneous Determination of Carbon Dioxide and Oxygen in Blood*

Hemolytic agent used	CO <sub>2</sub> per liter blood		O <sub>2</sub> per liter blood		Per cent of result with Kahlbaum saponin	
	Replicates	Mean	Replicates	Mean	Mean CO <sub>2</sub>	Mean O <sub>2</sub>
	mm	mm	mm	mm		
Kahlbaum saponin	16.82		10.29			
	16.69		10.31			
	16.75	16.75	10.25	10.28	100.0	100.0
Senega root	16.77		10.33			
	16.78	16.78	10.32	10.33	100.1	100.5
Root soapwort	16.84		10.38			
	16.94	16.89	10.28	10.33	100.8	100.5

TABLE III

*Determination of Carbon Monoxide Capacity of Blood by Method of Van Slyke, Hiller, Weisiger, and Cruz (6), with and without Saponin*

Blood No.	Determination*	Hemolytic agent added to borax solution	Carbon monoxide capacity		Mean in per cent of results with Kahlbaum saponin
			Duplicates	Mean	
			vol. per cent	vol. per cent	
4	Total Hb	Kahlbaum saponin	22.04		
4	" "	None	21.94	21.99	100.0
			21.93		
4	" "	None, but blood shaken 2 min. with borax solution before saturating with CO	21.98	21.96	99.9
			22.00		
			21.98	21.99	100.0
5	Total Hb	Kahlbaum saponin	18.52		100.0
	" "	None	18.50		99.9
5	Active "	Kahlbaum saponin	18.24		100.0
	" "	None	18.24		100.0

\* "Total Hb" is determined from the CO capacity of blood treated with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>; "active Hb" from CO capacity without Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (6).

1 ml. samples of blood were analyzed and readings of carbon dioxide were taken with the meniscus at the 2 ml. mark, and of oxygen at the 0.5 ml. mark.

The results when senega root or root soapwort was used as a substitute for saponin differed by less than 1 per cent from the results obtained with the Kahlbaum saponin, as shown in Table II.

*Determination of Carbon Monoxide Capacity of Blood Without Saponin*—Hemoglobin was determined in blood by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz (6), with and without addition of Kahlbaum saponin to the borax solution. The results in Table III show that a hemolytic agent is not required for the procedure.

#### DISCUSSION

When urea as hemolytic agent in the determination of oxygen in blood is added simultaneously with the ferricyanide, as is saponin in the method of Van Slyke and Neill (1), low results are obtained, as shown in Table I. If, however, the blood is first laked in the urea solution before adding potassium ferricyanide, good results can be obtained. Blanks performed by this procedure, however, are high (10 mm. of  $P_{O_2}$  at the 0.5 ml. mark), and if such blanks are used the results are lowered. It was found, however, that when a protein, such as egg albumin, was added to the urea solution before addition of potassium ferricyanide, the blanks were lowered to 3 mm. of  $P_{O_2}$  at the 0.5 ml. mark. When egg albumin in concentration of 1 per cent was added to 40 per cent urea solution, and both blood and blank analyses were performed by the procedure as here described, the results obtained were comparable to those by the original procedure with a good saponin.

#### SUMMARY

Some commercial preparations of saponin currently obtainable, when used as hemolytic agents in the determination of oxygen in blood by the method of Van Slyke and Neill, give low results.

Infusions of senega root, root soapwort, and quillaja soap bark can be used as substitutes for saponin. Urea can be used in place of saponin if egg albumin is added to the urea solution and if the blood is laked in the urea-albumin solution *before* potassium ferricyanide is added.

In the determination of hemoglobin by the carbon monoxide capacity method of Van Slyke, Hiller, Weisiger, and Cruz, saponin can be omitted from the reagents without affecting the results. The successive actions of borax and acetate buffer appear to lake the cells sufficiently to make the HbCO completely accessible to the ferricyanide.

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# INTERACTIONS OF QUATERNARY AMMONIUM COMPOUNDS AND PROTEINS

## A SIMPLE METHOD FOR THE RAPID ESTIMATION OF URINARY PROTEIN CONCENTRATIONS WITH ALKYL-DIMETHYLBENZYL-AMMONIUM COMPOUNDS\*

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(Received for publication, July 15, 1948)

A simple method of titration with a cationic detergent has been developed which permits rapid estimation of the concentration of urinary protein with a minimum of manipulations and time. The method depends on the formation of an insoluble anion-cation complex between quaternary ammonium ions, hereinafter referred to as the cationic detergent, and proteins, in the present instance mainly human albumin, at alkaline pH. No protein is precipitated from solution by the addition of the cationic detergent until a slight excess of this is present, at which time the solution shows faint but definite turbidity. Addition of more cationic detergent results at first in increasing turbidity, but as the addition is continued the turbidity decreases, almost as abruptly as it appeared, to give a nearly clear solution. Further addition of the cationic detergent is not attended by reappearance of turbidity. The end-point used in the present method is the appearance of a definite turbidity. The protein-quaternary ammonium ion complex formation is sensitive to ionic strength, non-electrolyte concentration, pH, and type of protein present (see, for example, Polonovski and Macheboeuf (1), Schmidt (2), and Valko (3)). For this reason, the method is restricted for accurate results to urine protein of the order of 2 or more gm. per liter (0.4 or more mg. of protein per sample) in order that the dilutions made may be sufficient to reduce the factors of urine ionic strength, non-electrolytes, and pH to negligible proportions. The method is, however, applicable to most cases of significant proteinuria. The simplicity of the procedure is indicated by the fact that we have been able to complete nearly 200 analyses in an afternoon.

If the end-point is determined by the optical density change in a photometer, the method can be used for samples containing as little as 0.02 mg. of protein.

\* The preliminary phases of this work were started while the author was a Fellow in the Medical Sciences of the National Research Council in 1945-46.

### Reagents

*Alkyldimethylbenzylammonium chloride*,<sup>1</sup> 0.1 per cent solution in distilled water. The solutions have been found to be stable for at least 3 months. This solution will be referred to as "the detergent."

2 *N* NaOH.

### Procedure

*Preparation of Standard Curve Relating Amount of Added Detergent to Amount of Protein in Sample*—Because of the uncertain composition of the detergents available it is essential that a standard curve be prepared relating the amount of added detergent to the amount of protein in the sample. For the present work a series of twelve urine samples was selected for use as a standard. The protein concentration of the urines was calculated from the difference between total nitrogen and nitrogen after precipitation of the proteins with an equal volume of 10 per cent trichloroacetic acid. The technique used for digestion and distillation was that described by Hiller, Plazin, and Van Slyke (4). Turbidity titrations were then carried out as described below; the results were plotted with the protein concentration as ordinates and the ml. of detergent used as abscissae. An example of a standard curve with human serum albumin is given in Fig. 1.

*Titration of Urine Proteins*—0.1 or 0.2 ml. of the urine samples containing 0.4 or more mg. of protein is accurately pipetted into 2.5 × 10 cm. test-tubes, and 4 ml. of distilled water are added, followed by 0.2 ml. of 2 *N* NaOH. (The pH of the solution is approximately 13.) The detergent is then added from a 2 ml. burette, with swirling, until the appearance of a definite and permanent turbidity. This turbidity is most easily detected by titrating against a black background and by having a beam of light passing through the test-tube at right angles. Reproducibility may be insured by comparing with samples of the standards titrated previously, but after a few preliminary trials it will be found that the reproducibility of this

<sup>1</sup> Unless otherwise specified, the results reported here have been obtained with myristamidopropyldimethylbenzylammomium chloride (trade name, Aerosol M), manufactured by the American Cyanamid Company, 30 Rockefeller Plaza, New York 20. For use 1 ml. of the concentrated detergent is diluted to 500 ml. in distilled water. We are indebted to the American Cyanamid Company for several samples of their product. Equally satisfactory and similar results have been obtained with cetyldimethylbenzylammonium chloride and dodecyldimethylbenzylammonium chloride in 0.1 per cent solutions. These last two compounds are available from the Onyx Oil and Chemical Company, Jersey City 2, New Jersey. A mixture of alkyldimethylbenzylammonium chlorides (alkyl = C<sub>8</sub>H<sub>17</sub> to C<sub>18</sub>H<sub>37</sub>), manufactured by the Winthrop Chemical Company, Inc., 170 Varick Street, New York 13, under the trade name Zephiran, may also be used in 0.1 per cent concentration, though it is slightly less satisfactory.

turbidity end-point is no more difficult than in ordinary acid-base titrations with phenolphthalein as an indicator. The mg. of protein present in the sample analyzed are then calculated from a standard curve, established from urines containing varying amounts of protein calculated from Kjeldahl determinations.

*Calculations*—A standard curve relating ml. of detergent to protein concentration is prepared as described above. The results for unknowns are read off the curve and the proper correction made for dilution of the urine.

### Results

*Standard Curve with Human Serum Albumin; Reproducibility of Results*—Fig. 1 shows the relationship of the ml. of detergent required to reach the end-point and the mg. of human serum albumin present as determined by the Kjeldahl method. An average of six titrations was carried out for each point of the curve. The ml. of detergent added and the estimated standard deviations for each point were  $0.519 \pm 0.023$ ,  $0.863 \pm 0.012$ ,  $1.220 \pm 0.019$ ,  $1.528 \pm 0.011$ ,  $1.834 \pm 0.020$ ,  $2.224 \pm 0.022$ . The dotted line in Fig. 1 (the extension of the straight line through the points) shows that the true end-point was overstepped by an absolute number of ml. for each point on the curve. The overstepping is equal to the  $X$  intercept of the extended straight line. This overstepping, equivalent to 0.18 ml. of detergent solution, is due to the fact that the first appearance of turbidity was not taken as the end-point, but rather the appearance of a definite turbidity, the reproducibility of which is indicated by the standard deviations above.

*Comparison of Results Obtained by Turbidity Titration with Several Detergents by Biuret Reaction*—In Fig. 2 are plotted the protein concentrations of urine of patients with the nephrotic syndrome, some of whom were receiving large amounts of human serum albumin intravenously. The results obtained by turbidity titration with myristamidopropyltrimethylbenzylammonium chloride are plotted as ordinates, while the results obtained by the biuret reaction (5) and the Kjeldahl method (as described above under "Preparation of standard curve") are plotted as abscissae. Twelve Kjeldahl determinations of urine protein were used to calculate the equivalence of ml. of detergent and mg. of urinary protein. The estimated standard deviation of results by the turbidity titration from these by the Kjeldahl method was  $\pm 3.71$  per cent. When the turbidity titration results were calculated as per cent of the biuret results, the mean for the 105 estimations was 100.5 per cent, with an estimated standard deviation of  $\pm 4.8$  per cent.

In another smaller series of eleven urines the results of turbidity titrations with other alkyltrimethylbenzylammonium compounds were compared with results of the biuret reaction as applied by Hiller, Greif, and Beckman (5).

For myristamidopropyldimethylbenzylammonium chloride the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of  $\pm 4.45$  per cent; for cetyldimethylbenzylammonium chloride the mean was 100.6 per cent of the biuret results, with an estimated standard deviation of  $\pm 3.65$  per cent; for dodecyldimethylbenzylammonium chloride the

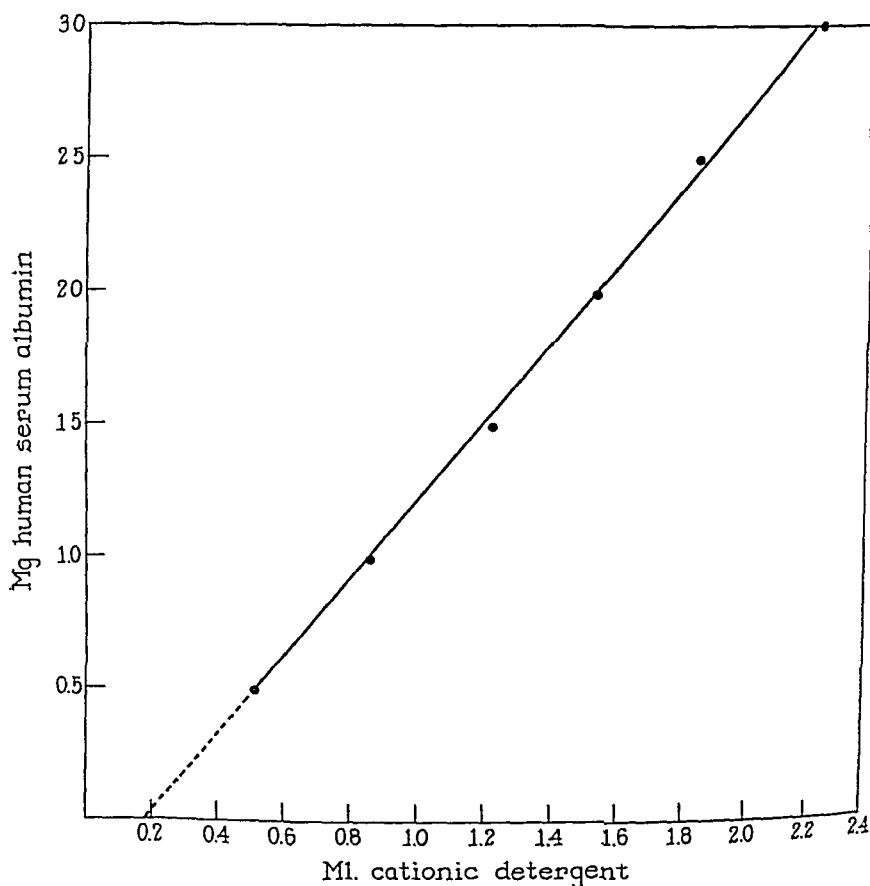


Fig. 1. "Standard curve" of human serum albumin titrated with myristamidopropyldimethylbenzylammonium chloride (1:500).

mean was 99.6 per cent of the biuret results, with an estimated standard deviation of  $\pm 4.59$  per cent; and for Zephiran the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of  $\pm 5.56$ .

*Relation of Optical Density to Amounts of Detergent Added to Solutions of Albumin*—Fig. 3 shows the result of adding increasing amounts of detergent solution to 0.2, 0.5, 1.0, and 1.5 mg. of human serum albumin under the

conditions described under "Procedure." The solutions were not made up to the same final volume, as it was desired to check the end-point used under the conditions of the estimations. The optical densities were measured in cylindrical cuvettes (12 × 75 mm. outside diameter) in a Coleman model

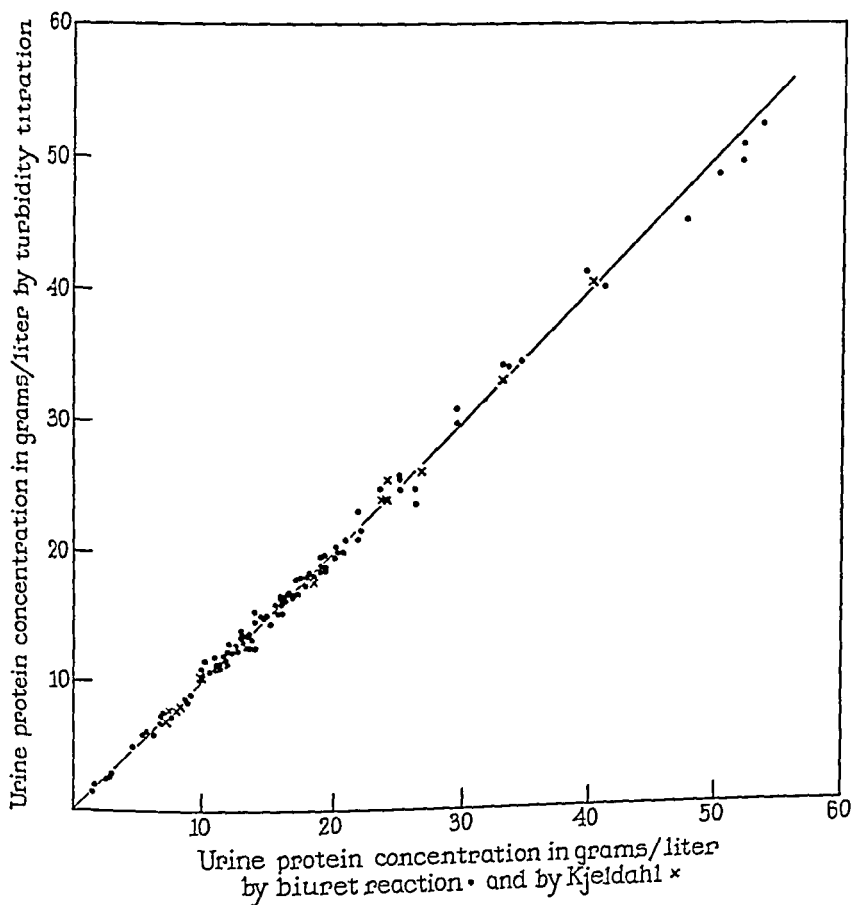


Fig. 2. Comparison of results obtained by turbidity titration with myristamido-propyldimethylbenzylammonium chloride (1:500) by the biuret reaction and the Kjeldahl method.

6 clinical spectrophotometer at  $\lambda = 450 \text{ m}\mu$ , within about 30 minutes of the addition of detergent to the first of a series. It will be seen that if an optical density of 0.025 is taken as the end-point (this is the approximate optical density used as the visual end-point for the data in Figs. 1 and 2) the number of ml. of detergent added to the sample containing the smallest amount of protein is not in proportion to the number of ml. added to

samples containing larger amounts of proteins. Therefore, if protein were calculated as directly proportional to the detergent added, one would somewhat overestimate the amount of protein present, especially for very small amounts of protein. That the estimated "equivalence" end-points are in reasonably good proportion (0.13, 0.30, 0.60, 0.90 ml. of detergent for 0.2, 0.5, 1.0, and 1.5 mg. of protein, respectively), however, confirms the stoichiometry of the procedure already demonstrated in Fig. 1.

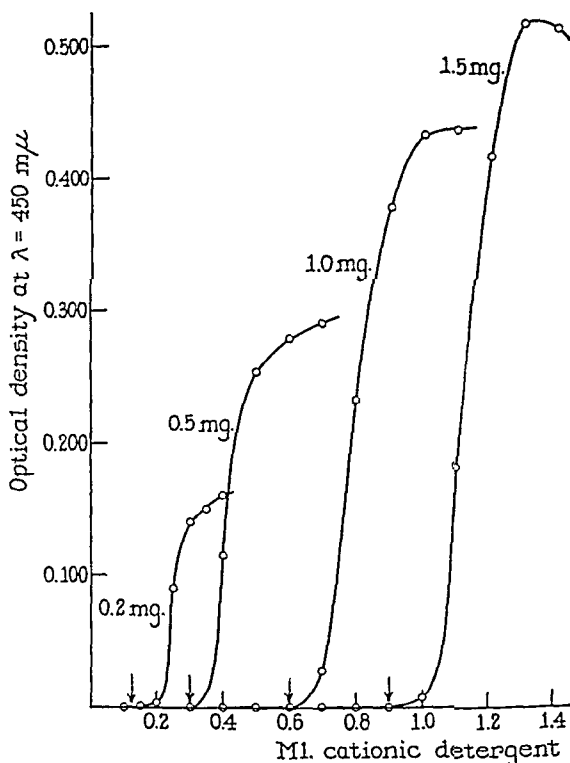


Fig. 3. Relation of optical density to amounts of detergent (myristamidopropyl-dimethylbenzylammonium chloride) added to solutions of human serum albumin. The arrows indicate the estimated "equivalence" end-points.

*Effect of pH*—Fig. 4 shows the optical density curves obtained by adding varying amounts of detergent to the same amount of protein, namely 1.0 mg. of human serum albumin, at pH 7.0, pH 7.8, pH 9.2, respectively, under the conditions described under "Procedure" (pH approximately 13). Addition of 1 ml. of 2 N NaOH instead of 0.2 ml. results in a very slight shift of the curve to the right.

*Other Detergents*—A few other cationic detergents have been tried, as



mentioned in foot-note 1. Dimethylbenzylcetylammonium chloride, dimethylbenzyl dodecylammonium chloride, and Zephiran give results similar to those obtained with the detergent routinely used as described above. The high molecular weight alkylimidazolinium compounds are not satisfactory for the present purposes because of the slowness of the development of the turbidity. Alkyltrimethylammonium compounds give indefinite end-points. Unsatisfactory results were also obtained with an N-

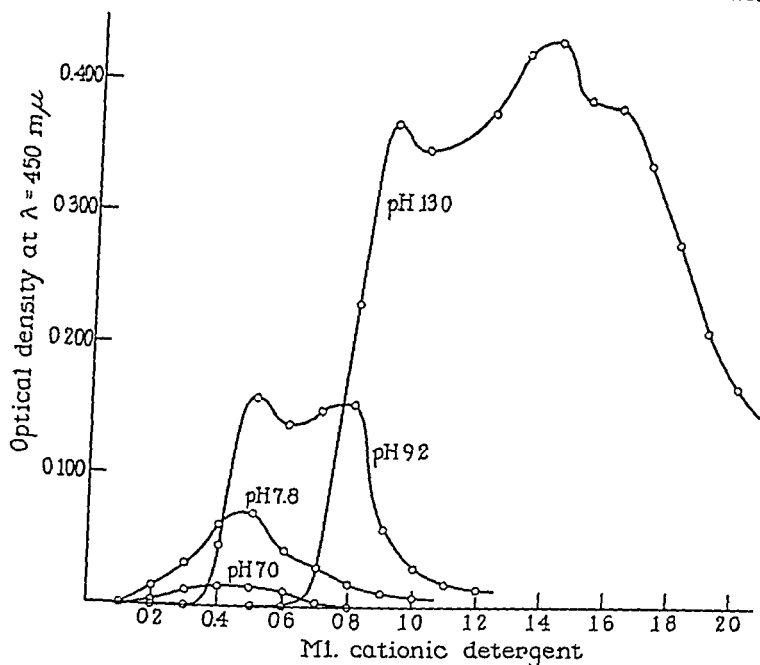


Fig. 4. Effect of pH on titration curves of human serum albumin with myristamidopropyl dimethylbenzylammonium chloride.

(acylcolaminoformylmethyl)pyridinium chloride, and with cetylpyridinium chloride.

*Relation of Optical Density to Amount of Detergent Added to Plasmas, with Differing Albumin and Globulin Concentrations*—The method is not at present recommended for plasma proteins, because they present some difficulties not encountered in urine. Because of the turbidity present before titration in some plasmas it is not possible in all cases to use the visual end-point described above to estimate total protein in plasma. In some preliminary experiments with plasmas of varying albumin and globulin concentrations the optical density curves show good correspondence between ml. of detergent added to reach the "equivalence" end-point and

total protein present. Peculiarities appear in the titration curves in the presence of abnormally large amounts of  $\gamma$ -globulins. Further work on the plasma proteins is under way.

#### DISCUSSION

While a considerable amount of work has been done on anionic and cationic detergent-protein complex formation, the exact nature of the process is not completely known. It is apparent from the work of Putnam and Neurath (6) that the cationic groups of proteins play a major rôle with anionic detergents at acid pH. Similarly at alkaline pH the negative charges of the protein molecules must play a major rôle with cationic detergents. It may be noted, however, that the detergents are protein denaturants (7) and under certain conditions act to accelerate protein hydrolysis (8). Further, there is probably an unfolding of certain proteins in the presence of increasing amounts of detergents; native proteins cannot always be recovered quantitatively after exposure to the detergents (6, 9).

For the present work, a tentative working hypothesis (an extension of that of Polonovski and Macheboeuf (1)) is as follows: At pH 13, the carboxyl, phenolic, and sulfhydryl groups are all dissociated; there are still some positively charged guanidine groups, but the charges of the protein molecule are mainly negative. As detergent is added to the protein solution, the positively charged quaternary ammonium ions are attracted to the negative charges of the protein molecule by Coulomb forces, van der Waals forces playing a rôle dependent on the substituents of the nitrogen of the detergent. As more and more of the negative charges of the protein are "neutralized" by the positively charged quaternary ammonium ions, the protein molecule becomes less and less polar until finally, at the so called "equivalence" end-point, aggregation of the protein molecules becomes possible through van der Waals forces. With further addition of detergent the non-polar portions of the added detergent become associated through van der Waals forces with the non-polar portions of the detergent ions attached to the protein, the protein-detergent complexes become positively charged and repel each other, and dispersal of the aggregates then takes place. The N substituents in the detergent ions play a rôle in determining the extent of the van der Waals forces and may be responsible for the unsatisfactory end-points obtained with the alkyltrimethyl, alkyl dimethylethyl, alkylimidazolinium, and alkylpyridinium quaternary compounds. With the alkyl dimethylbenzyl compounds the number of moles of detergent required to reach the "equivalence" end-point (Fig. 3) per mole of human serum albumin varies with the detergent used, though it is of the order of magnitude of the sum of the total free carboxyl, phenolic, and sulfhydryl groups calculated from Brand's data (10). Chain length of the alkyl

group may be of importance in this. A similarity to the zone phenomenon of antigen-antibody titration is evident in the fact that a definite protein-detergent ratio is required to produce aggregation.

In any case, whatever the exact nature of the protein-detergent complex, it is evident that reasonably quantitative results are obtainable by the procedure herein described. A method, to which the present one is similar in principle, was recently introduced by Lambert (11) for the volumetric analysis of anionic and cationic detergents by turbidity titration. Maximum turbidity measured photometrically is used by Lambert as the end-point, rather than the appearance of a definite turbidity estimated visually. Use of maximum turbidity is possible for the estimation of proteins, but the additional equipment required and the extra time and manipulations involved would reduce the advantages of the present procedure. The technique described by Lambert takes about 5 times as long.

#### SUMMARY

A rapid simple method for the estimation of urinary protein concentrations is presented. The method depends on the formation of an insoluble complex between quaternary ammonium ions and proteins at pH 13. End-points are estimated visually by the appearance of a faint but definite and permanent turbidity. The standard deviation was  $\pm 3.71$  per cent from determinations by the Kjeldahl method in a series of twelve estimations. In a series of 105 estimations, the standard deviation was  $\pm 4.8$  per cent from determinations by the biuret reaction. Several types of quaternary ammonium compounds have been tried; the most satisfactory for the present purposes are the alkyltrimethylbenzylammonium group. A brief discussion of the possible mechanism of the quaternary ammonium protein anion complex formation is given.

It is a pleasure to acknowledge the help given by Dr. Hiller and Dr. C in making available their biuret procedure before its publication.

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# USE OF THE HYPOBROMITE REACTION FOR THE ESTIMATION OF AMMONIA PLUS UREA NITROGEN IN URINES CONTAINING LARGE AMOUNTS OF PROTEIN; THE REACTION OF ALKALINE HYPOBROMITE WITH PROTEINS

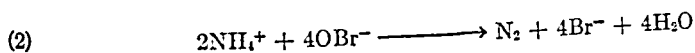
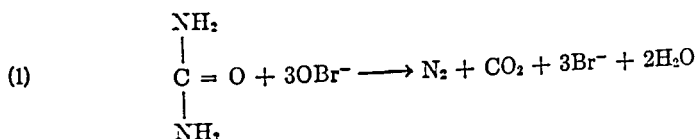
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(Received for publication, July 28, 1948)

Alkaline hypohalites have been in use for nearly a century for the estimation of urea or of urea and ammonia nitrogen in blood and urine. Alkaline hypochlorite, first used by Davy (1) in 1854, was replaced a few years later by alkaline hypobromite as a result of Knop's work (2). The  $N_2$  evolved in the following reactions was measured volumetrically.



After Knop's work there was a spate of modifications of the method, modifications having to do with concentrations of the reagents, length of reaction time, design of apparatus, elimination, and occasionally identification of interfering substances. These modifications finally culminated in the manometric procedure of Van Slyke (3) as modified by Van Slyke and Kugel (4). This method has been extensively used both in this laboratory and elsewhere for the estimation of the blood and urine urea nitrogen from which, with knowledge of the urine flow, the urea clearance can be calculated.

While reasonably satisfactory when only approximate results are required, the hypobromite methods all suffer from the fact that the hypobromite reaction is neither specific nor quantitative. Many other substances besides urea and ammonia evolve nitrogen; some, such as guanidine and mono-substituted guanidines, give off nitrogen in considerable amounts; others, such as amino acids, amines, and peptides, in smaller amounts. Proteins evolve  $N$  in amounts determined chiefly by their arginine content. In addition, glucose in the high concentrations occasionally present in the urine of diabetics can cause error (5, 6).

Under the conditions of blood or plasma analyses, the hypobromite liberates approximately 98 per cent of the urea nitrogen; under the conditions of the urine analyses, hypobromite liberates approximately 95 per cent of the urea and ammonia nitrogen (3, 4). In the case of blood or plasma, the presence of non-urea N-evolving substances in the filtrates requires that a subtractive correction be applied to the calculated results; in the case of urine, one depends on the evolution of nitrogen from non-urea or ammonia substances to compensate approximately for the deficit of 5 per cent in the nitrogen evolved from the urea and ammonia (3). In some cases, however, and especially when protein is present, the evolution of nitrogen instead of approximately compensating may introduce a positive error as high as 30 per cent. While it is possible to correct approximately for the protein error if the protein concentration in the urine is known, it is preferable to remove the proteins before doing the analyses.

It is the purpose of the work reported here to present some additional data on possible sources of error inherent in the hypobromite procedure as at present applied (3, 4) and to describe an obvious means of circumventing the protein error. In addition, the reaction of alkaline hypobromite with certain proteins and compounds will be described briefly.

### *Removal of Proteins from Urine*

The zinc hydroxide procedure of Somogyi (7) is used because it has been found to be effective in removing some of the interfering substances of blood as well as proteins (4).

### *Reagents*

*Acid zinc sulfate solution.* 12.5 gm. of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  are dissolved in 125 ml. of 0.25 N  $\text{H}_2\text{SO}_4$  and diluted to 1 liter. This solution is used for the precipitation of proteins from whole blood. For urine and plasma 100 ml. of this solution are diluted to 212 ml. with water.

*0.75 N NaOH.* When 50 ml. of the zinc sulfate solution for whole blood are titrated with 0.75 N NaOH, with phenolphthalein as indicator, 6.7 to 6.8 ml. of the alkali should be required. The solution is shaken vigorously during the titration.

### *Procedure*

For urine containing less than 80 gm. of protein per liter, 1 ml. of urine is pipetted into a centrifuge tube, and 8.5 ml. of the zinc sulfate diluted for urine or plasma are added, followed by 0.5 ml. of the 0.75 N NaOH. The tube is stoppered, shaken vigorously, and allowed to stand for 10 minutes. It is then centrifuged for 10 minutes at 2000 R.P.M. or more (18 cm. radius). The supernatant solution is then filtered through a pledget

of washed cotton placed in the stem of a funnel, and aliquots are taken for analysis. A blank analysis is run on 0.9 per cent NaCl to correct for the non-urea nitrogen-liberating substances in the cotton and the reagents.

For urine containing 80 or more gm. of protein per liter, 0.5 ml. samples of urine are taken, and 0.5 ml. of distilled water is added to each. The diluted urines are then treated as above.

Details concerning manometric determination of urea in the filtrates and the factors used in calculations are given in the original publications (3, 4).

TABLE I  
*Comparison of Nitrogen Evolved from Urines Containing Protein and Protein-Free Filtrates of Same Urines*

Urine No.	N before protein removal (a)	N after protein removal (b)	(a) - (b)	(a) as per cent of (b)	Protein concentration
	gm. per l.	gm. per l.	gm. per l.		gm. per l.
1	2.338	1.908	0.430	122.6	57.5
2	0.734	0.606	0.128	121.1	17.3
3	2.038	1.574	0.464	129.6	59.5
4	2.250	1.908	0.342	118.0	35.0
5	6.050	5.710	0.340	106.0	35.0
6	5.340	5.162	0.178	103.4	28.0
7	0.621	0.487	0.134	127.6	15.0
8	2.568	2.481	0.087	103.6	12.0
9	5.635	5.371	0.264	104.9	36.0
10	7.850	7.468	0.382	105.1	60.5
11	4.829	4.680	0.139	103.2	18.0
12	1.965	1.882	0.083	104.4	6.7
13	7.100	6.780	0.320	104.7	39.5
14	2.937	2.858	0.079	102.8	3.6
15	7.278	7.175	0.103	101.4	4.6
16	5.418	5.198	0.220	104.2	18.0
17	2.803	2.698	0.105	103.9	10.4
18	2.051	1.915	0.146	107.1	38.6
19	4.714	4.554	0.160	103.5	13.0
20	7.108	6.823	0.185	104.1	27.9

*Comparison of N Evolved from Urine Containing Protein and from Protein-Free Filtrates of Same Urines*

Table I shows the differences in N evolved from urines containing protein and protein-free filtrates of the same urines. The urines were obtained from patients with the nephrotic syndrome, some of whom were receiving large doses of human serum albumin intravenously. Generally speaking, unless the urines are from patients receiving serum albumin or plasma

intravenously, urine protein concentrations will not exceed 25 to 30 gm. per liter, provided the urine flow is reasonably high (1 or more ml. per minute). If, however, the patient's urine flow is small (less than 1 ml. per minute), then the urine protein concentration may rise to 40 or 50 gm. per liter. The urine protein concentrations were calculated from nitrogen determinations done by the micro- or macro-Kjeldahl procedures of Hiller, Plazin, and Van Slyke (8). The total nitrogen was determined directly, the non-protein nitrogen was determined after removal of the proteins by

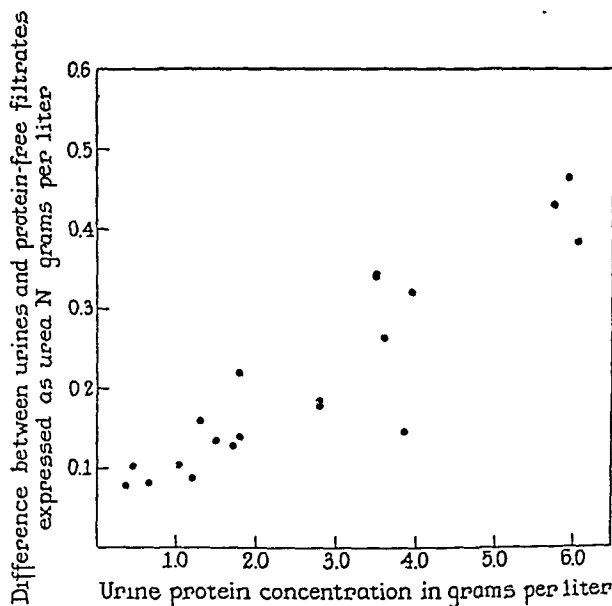


FIG. 1. Relationship of concentration of urine protein to difference in N evolved from urines and protein-free filtrates.

precipitation with equal volumes of 10 per cent trichloroacetic acid, and the protein nitrogen was calculated from the difference. It will be noted that the errors due to protein are all positive and vary from +1.4 per cent to +29.6 per cent. The magnitude of the percentage error is determined by the ratio of the protein N evolved by hypobromite to the N evolved from the urea plus ammonia, not by the absolute amount of protein present. Fig. 1, in which protein concentration is plotted against the difference in N evolved before and after removal of the proteins, shows that there is considerable scattering. A correction made on the basis of the data of Table I would be approximate, though adequate for clinical purposes. Fig. 1 is to be compared with Fig. 2 which shows, from analyses of pure



albumin solutions, the relatively close proportionality of N evolved to albumin present under the conditions of routine urine analyses when other N-evolving substances are absent. An additional point is that, in protein-free urine from normal individuals, treatment of the urine with the Somogyi reagents resulted in lower values for urea plus ammonia N than in untreated urine; this effect is presumably due to precipitation of interfering substances other than protein.

It may be noted here that the reaction of alkaline hypobromite with proteins and amino acids is not restricted to the guanidino group of the arginine; tyrosine is brominated, free amino groups react, and there is also reaction with peptide groups (see, for example, Goldschmidt *et al.* (9, 10)). These reactions occur with little or no evolution of N. The result is that much more hypobromite is used up in reaction with proteins than would be expected from the N liberated. This fact becomes of importance when the protein concentrations are very high, because the reaction with protein may not leave enough hypobromite to give the expected N yield from urea. This effect has been found in a few cases in which the urine was not sufficiently diluted before analysis; less N was evolved per aliquot of urine than after greater dilution. If protein-free filtrates are used, error from this effect of excess protein is avoided.

#### *Evolution of N from Various Proteins*

The evolution of N from human serum albumin was studied in some detail. Under the conditions of the routine urine analyses (3, 4) (2.5 minutes reaction time measured from the moment of addition of the alkaline hypobromite to the moment the solution was brought to the 2.0 ml. mark of the gas chamber for reading the volume), a reasonably stoichiometric relationship was found between mg. of N evolved and mg. of protein present, as illustrated in Fig. 2. It was found, however, that if the reaction time was prolonged beyond 2.5 minutes more nitrogen was evolved, though at a much slower rate than during the first 2.5 minutes. An example of the time course of the reaction is given in Fig. 3 for 20.0 mg. of human serum albumin (Curve A), and for 18.35 mg. of bovine  $\gamma$ -globulin (Curve B). Similar curves were obtained with edestin and gelatin.

Because arginine is the only guanidine derivative reported in proteins in appreciable amounts, it was thought that the N evolved from intact proteins by alkaline hypobromite might give a measure of the arginine content of those proteins. It was found, however, in the few proteins examined that less N was evolved than was calculated from the arginine contents. While the results were quite reproducible provided the reaction times were the same, in no case was the calculated amount of N evolved. The data in Table II were obtained from proteins in aqueous solutions of

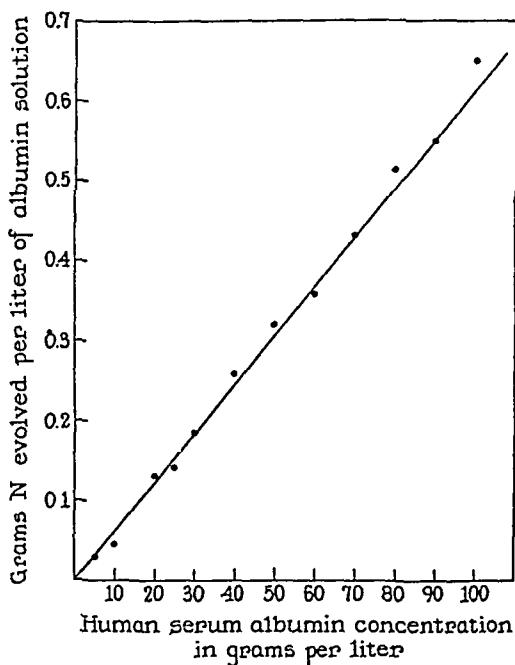


FIG. 2. Evolution of N from various amounts of human serum albumin in 2.5 minutes.

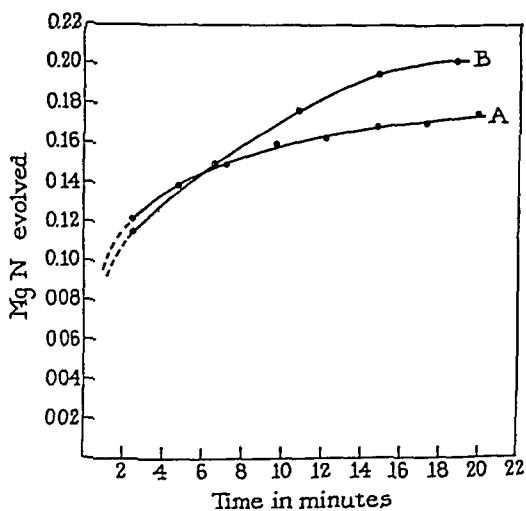


FIG. 3. Evolution of N from 20.0 mg. of human serum albumin (Curve A) and from 18.36 mg. of bovine  $\gamma$ -globulin (Curve B).

approximately 1 per cent concentration, and the results were calculated on the basis of total protein N as determined by macro-Kjeldahl analysis (8). The values for arginine N content were taken from tables published by Chibnall (11) and Brand (12) and were not determined by analysis of the protein preparations used in these experiments; for this reason the data should be considered as preliminary.

TABLE II  
*Evolution of Nitrogen from Various Proteins by Alkaline Hypobromite*

Protein	Protein N in sample (a)	Calculated arginine N in sample (b)	Calculated N evolvable by hypobromite* (c)	N evolved by hypobromite in 2.5 min (d)	N evolved as per cent of (c)
	mg.	mg	mg	mg	
Human serum albumin. . . . .	3 190	0.3990	0 1894	0 1220	64.4
Gelatin. . . . .	3 402	0 5175	0.2459	0 2373	92.9
Edestin . . . . .	3 404	0 9820	0 4661	0 3745	80.3

\* Calculated N corrected for 5 per cent deficit in N evolved from urea and arginine under the conditions used for the analysis. Calculated arginine N is based on data of Brand (12) for human serum albumin. For gelatin and edestin, calculations are based on data assembled by Chibnall (11).

*Evolution of N by Hypobromite Reaction from Guanidine, Guanidine Derivatives, Amino Acids, and Other Compounds*

Under the conditions of the routine urinalysis, many substances besides proteins were found to evolve N when they reacted with alkaline hypobromite. In particular, guanidine and its derivatives of the type  $R_1R_2N-C(=NH)-NH_2$  (where  $R_1$  and  $R_2$  may be H, alkyl, or aryl groups) give off nearly two-thirds of their guanidine nitrogen. Of such a type are, for example, arginine, methylguanidine, creatine, and guanidoacetic acid, and of a similar type are dicyandiamide and guanylurea. The evolution of N from the guanidino groups is quite rapid with these compounds and is complete or nearly complete in the 2.5 minutes required for urine analysis. Another group of substances, aliphatic diamines, evolve N at a slower but still appreciable rate. Pentamethylene- and hexamethylenediamine, ornithine and lysine evolve about 3 per cent of their total nitrogen in 2.5 minutes, and about 10 per cent of their total nitrogen in 10 minutes. This evolution of nitrogen may stem from nitrile formation, ring closure to form an amidine, and reaction of this latter group with more hypobromite. In contrast, monoamino-, mono-, or dicarboxylic acids, asparagine, glutamine, and creatine evolve practically no N in the routine reaction time. Glutathione evolves slightly less N per mole than do the

diamines. Mono-N-substituted ureas evolve nearly one-half their total urea nitrogen.

In addition to the above nitrogen compounds, glucose also evolves gas which is measured as N in the routine analyses. A 1 per cent solution of glucose evolves an amount of gas equivalent to approximately 0.024 gm. of N per liter. Hence the hypobromite method should not be employed for analyses of urine from diabetics.

#### DISCUSSION

The sources of error and their significance are apparent from the above paragraphs. Of ancillary interest is the finding that not all of the arginine guanidino groups in proteins appear to be available for reaction with alkaline hypobromite under the condition used; this suggests that some of these groups may be involved in linkages and are therefore not free. (There is little likelihood that this effect is due to exhaustion of the  $\text{OBr}^-$  because of the proportionality of the results with different amounts of protein. The expected N is evolved from protein-urea mixtures.) Similar observations have been made in the case of egg albumin by Goldschmidt *et al.* (9); in this protein even after a 4 hour exposure to hypobromite, there was still some intact arginine which could be demonstrated, suggesting that the arginine linkages in proteins were not all identical and that some of the guanidino groups were protected. Of similar import are the observations of Roche and his collaborators (see, for example, (13)) who have used the Sakaguchi reaction on intact proteins and found less color developed than would be expected from the total arginine content of the proteins examined. In addition, Simms has suggested, from the evidence offered by his titration curves of certain proteins, that some of the arginine guanidino groups are somehow linked to other portions of the protein molecule (14). Further work is under way at present on the reaction of hypobromite with proteins and guanidine derivatives; preliminary experiments suggest that the hypobromite reaction may be of some use for rapid semimicroestimation of arginine in protein hydrolysates (*cf.* (15)).

#### SUMMARY

Under the conditions used for gasometric determination of urea and ammonia by the hypobromite reaction, the greater part of the guanidino groups in proteins reacts with evolution of nitrogen gas. In urine with high protein concentration (*e.g.* 30 or more gm. per liter) the nitrogen gas evolved from the proteins may cause a plus error of as much as 30 per cent in the urea determination. This error can be prevented by preliminary removal of the proteins by Somogyi's zinc hydroxide precipitation.

The reaction of hypobromite with human serum albumin, bovine  $\gamma$ -

globulin, edestin, and gelatin has been studied with regard to its time course and evolution of total nitrogen gas. The final amounts of nitrogen gas evolved were from 64 to 93 per cent of that which would be evolved by the guanidino groups of arginine in the amounts reported to be present in these proteins. It appears that some of the guanidino groups in the protein molecules are not free to react with alkaline hypobromite.

The reactions of other substances with hypobromite have been reviewed. Glucose, if present in more than 2 per cent concentration, will cause evolution of enough gas to produce a significant positive error in determination of urine urea.

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## LETTERS TO THE EDITORS

### THE USE OF LACTOBACILLUS LEICHMANNII IN THE MICROBIOLOGICAL ASSAY OF THE "ANIMAL PROTEIN FACTOR"

Sirs:

During the course of isolation studies on the "animal protein factor" we have employed a microbiological assay method to follow its separation and purification from various naturally occurring crude materials. Specific

Concentrate per tube	0.1 N acid produced
$\gamma$	ml.
0	3.20
0	3.30
0.005	4.45
0.010	5.65
0.015	6.80
0.020	8.65
0.030	10.50
0.050	11.40

*Medium:* The 100 ml. double strength medium contains 1.0 gm. of acid-hydrolyzed norit-treated casein (*Univ. Texas Pub.*, No. 4137, 82 (1941)); 20 mg. each of tryptophan and cystine; 1 mg. each of adenine, guanine, xanthine, and uracil; 1 ml. each of Salts A, Salts B (Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 139, 675 (1941)); 1.2 gm. of sodium acetate; 4 gm. of glucose; 1  $\gamma$  of biotin; 400  $\gamma$  of pyridoxine; 400  $\gamma$  of pyridoxal; 200  $\gamma$  each of riboflavin, thiamine, pantothenic acid, nicotinic acid; 100  $\gamma$  of folic acid; 0.2 ml. of Tween 80 and 500 mg. of norit-treated tryptic digest of casein. (The casein digest is prepared as follows: 25 gm. of Labco casein are suspended in 250 ml. of 0.8 per cent  $\text{NaHCO}_3$  and incubated under benzene with 0.5 gm. of trypsin for 48 hours. After digestion, the material is autoclaved 15 minutes and filtered. The filtrate is taken to pH 2.0, stirred 1 hour with 10 gm. of norit A, and filtered. The pH of the filtrate is then adjusted to 6.6 to 6.8.) *Inoculum:* 0.1 ml. of 24 hour culture of *L. leichmannii* grown in 1 per cent tryptose milk tube suspended in 10 ml. of saline. *Sterilization:* Autoclave 15 minutes at 120°. *Incubation:* 37° for 24 hours for turbidimetric determination or 72 hours for titrimetric determination.

data showing the excellent correlation obtained between the microbiological and mouse growth<sup>1</sup> assay methods for the "animal protein factor" will be published at a later date.

<sup>1</sup> Bosshardt, D. K., Paul, W. K., O'Doherty, K., Huff, J. W., and Barnes, R. H., Abstracts, American Chemical Society, 113th meeting, 22C (1948).

The organism employed in the microbiological assay is *Lactobacillus leichmannii* (ATCC 4797). Its response to a highly purified preparation, prepared in these laboratories, demonstrated to have "animal protein factor" activity in the mouse, is shown in the table. Using the basal medium and the conditions described in the table, we routinely have obtained highly satisfactory assay results with *L. leichmannii* for over a year.

Recent reports from the Merck laboratories<sup>2</sup> have indicated that the "animal protein factor" is identical with or closely related to vitamin B<sub>12</sub>. If such is the case, it is probable that *L. leichmannii* may be the organism of choice for the microbiological assay of vitamin B<sub>12</sub>. The complex growth requirements of *L. lactis* (ATCC 8000), currently employed as the assay organism for vitamin B<sub>12</sub>,<sup>3</sup> have made its use in routine procedures difficult and unpredictable. We have found that both organisms will respond to purified liver preparations (shown to be active as "animal protein factor" in mice) and similarly to relatively large amounts of thymidine.<sup>4</sup> The effects of ascorbic acid and air, as reported by Shive *et al.*<sup>5</sup> for *L. lactis*, have been demonstrated with *L. leichmannii*. Autoclaving the tests for 15 minutes at 120° minimized the effects of ascorbic acid and air, but the growth stimulation by thymidine must be considered in the interpretation of results, particularly when crude materials are assayed.

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Received for publication, August 25, 1948

<sup>2</sup> Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).

<sup>3</sup> Short, M. S., *Science*, **107**, 396 (1948).

<sup>4</sup> Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, in press.

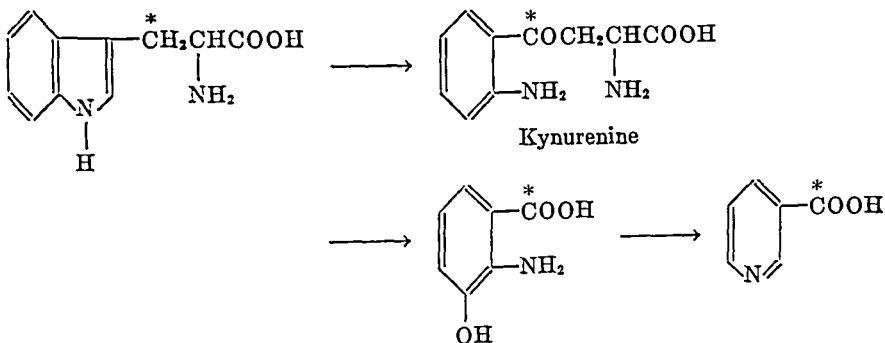
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# CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO NICOTINIC ACID\*

Sirs:

The mechanism of the conversion of tryptophan to nicotinic acid involving kynurenine and 3-hydroxyanthranilic acid as intermediates, has been demonstrated in *Neurospora*,<sup>1</sup> and confirmatory feeding experiments



in rats have been reported recently.<sup>2</sup> We have shown<sup>3</sup> that tryptophan- $\beta$ -C<sup>14</sup> is directly converted into kynurenine in the intact mammal, but, because the labeled carbon atom was lost, it was necessary (by other means) to establish its further conversion to nicotinic acid.

Compound	Specific activity, counts per min. per mg.
Tryptophan (fed).....	2740
N-Methylnicotinamide picrate (carrier).....	0.75
Nicotinic acid.....	2.0 (2.2 calculated)
Barium carbonate from decarboxylation.....	1.3 (1.2 " )

We have now synthesized<sup>4</sup> DL-tryptophan-3-C<sup>14</sup>, and have shown that the conversion proceeds in the same fashion in the rat as in *Neurospora*.

\* This paper is based on work performed under contract No. W-7405-eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley. We are also indebted to the Rockefeller Foundation for support.

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<sup>4</sup> Heidelberger, C., unpublished material.

The tryptophan (226 mg.) was fed to three rats by stomach tube, and the 24 hour urines were passed through a permutit column. The N-methylnicotinamide (750  $\gamma$ ) was eluted with KCl,<sup>5</sup> 76 mg. of carrier N-methylnicotinamide chloride were added, and the picrate was formed and purified. The amide was freed of picric acid and converted with HCl in a sealed tube into nicotinic acid, which was purified and decarboxylated catalytically. The specific activities are shown in the table. A significant observation is that the carbon atom, which is a precursor to the carboxyl group of the hydroxyanthranilic acid, becomes the carboxyl carbon of nicotinic acid.

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Received for publication, September 24, 1948

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<sup>5</sup> Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **158**, 265 (1945).

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# THE EFFECT OF DISSOCIATION IN *LACTOBACILLUS LACTIS* CULTURES ON THE REQUIREMENT FOR VITAMIN B<sub>12</sub>\*

Sirs:

Although *Lactobacillus lactis* Dorner (American Type Culture Collection No. 8000) is stable in its requirement for both vitamin B<sub>12</sub> (LLD factor) and the tomato juice (TJ) factor when cultured on a tomato juice-yeast extract-skim milk medium, the culture undergoes dissociation<sup>1</sup> when serial transfers are made on the usual yeast extract-glucose agar, with or without added tomato juice. Variants produced on this stock agar may grow in the amino acid assay medium with the tomato juice supplement only, and one isolated variant required no supplement when the concentration of the amino acids was doubled. L-Histidine was responsible for a large amount of this growth.

The medium used in the assay of the LLD factor<sup>2, 3</sup> and in connection with the isolation of vitamin B<sub>12</sub><sup>4</sup> is that of Baumgarten *et al.*,<sup>5</sup> modified by the addition of all the B vitamins at 10-fold concentration, except biotin and pteroylglutamic acid, which are used at 0.05  $\gamma$  per 10 ml. Adenine, guanine, uracil, and xanthine are added at 0.1 mg., and 0.5 ml. of clarified tomato juice (TJ factor) is used routinely. An extremely small inoculum of twice washed culture is used.

The assay as outlined above works well with the stabilized culture and purified liver extracts or crystalline vitamin B<sub>12</sub>, but erratic results may be obtained with the dissociating culture or with some crude materials because of the presence of inhibitory substances.<sup>2, 6</sup> The inhibition has been found to be due, in part, to high concentrations of folic acid. High levels of serine, *p*-aminobenzoic acid, xanthine, MnSO<sub>4</sub>, NaCl, and FeSO<sub>4</sub>, under certain conditions, also inhibit growth.

Culture filtrates of *Lactobacillus casei*, *Streptococcus faecalis* R, and

\* Scientific paper No. A214. Contribution No. 2134 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry). This study was supported in part by a grant from Merck and Company, Inc., Rahway, New Jersey.

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*Escherichia coli* and fresh chicken droppings (frozen for 24 hours) have a low vitamin B<sub>12</sub> activity.

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# RESPONSE OF LACTOBACILLUS LEICHMANNII 313 TO THE ANTIPERNICIOUS ANEMIA FACTOR

Sirs:

Recent observations <sup>1,2</sup> have shown that thymidine has a growth-stimulating effect upon *Lactobacillus lactis* Dorner under conditions in which growth is also promoted by vitamin B<sub>12</sub>.<sup>3,4</sup> Thymidine has also been found to promote the growth of *Lactobacillus leichmannii* 313.<sup>5</sup> Ac-

Addition per ml. basal medium		Optical density
	<i>mγ</i>	
None		0.06
APA factor	0.0005	0.04
	0.0015	0.06
	0.005	0.12
	0.015	0.20
	0.05	0.37
	0.15	0.62
	0.5	0.97
	1.5	1.17
	5.0	1.17
	<i>cc. mm.</i>	
Liver extract, injectable	0.005	0.17
	0.015	0.34
	0.05	0.60
	0.15	0.86
	0.5	1.17

cordingly, the effect of a sample of crystalline antipernicious anemia factor (APA factor) was tested with this organism, with a basal medium similar to that described by Snell and coworkers.<sup>5</sup> A final volume of 2 or 4 ml. and an incubation temperature of 37° were used. Readings were made at 14 hours with the results given in the table.

In other experiments higher levels of liver extract did not produce growth in excess of the maximum growth obtained with the APA factor. Half maximum growth was obtained with 0.25 to 0.5 γ of thymidine per

<sup>1</sup> Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).

<sup>2</sup> Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, **175**, 475 (1948).

<sup>3</sup> Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).

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<sup>5</sup> Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, **175**, 474 (1948).

ml.<sup>6</sup> When pteroylglutamic acid and *p*-aminobenzoic acid were omitted from the culture medium, the organism did not respond to the APA factor. Increasing the level of pteroylglutamic acid up to 50  $\gamma$  per ml. of culture medium did not permit growth in the absence of the APA factor.

The results indicated that *Lactobacillus leichmannii* 313 may be a sensitive test organism for the crystalline antipernicious anemia factor, and that liver extract produced a similar response.

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Received for publication, September 29, 1948

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<sup>6</sup> Samples of thymidine were kindly furnished by Dr. W. Shive and Dr. D. W. Woolley.

# THE SITE OF CONVERSION OF CAROTENE TO VITAMIN A\*

Sirs:

In a recent paper<sup>1</sup> from this department evidence was presented that one site of conversion of carotene to vitamin A in the rat was the intestinal wall. Since the analytical method used in these experiments was the Carr-Price reaction which is not specific for vitamin A, it was felt that the qualitative identification of vitamin A in the intestinal wall of the rat would strengthen the conclusions drawn. The purpose of this paper is to present such evidence.

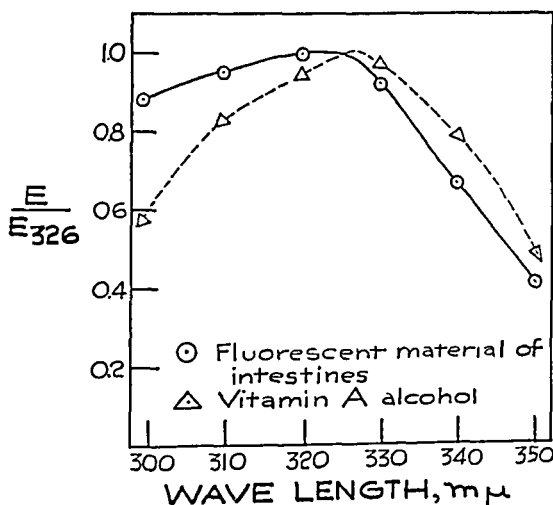


FIG. 1. The absorption curves of vitamin A alcohol and the fluorescent material of the intestinal tract.

Ten vitamin A-deficient rats were given 0.5 ml. of cottonseed oil containing 1200  $\gamma$  of carotene<sup>2</sup> and 0.5 per cent tocopherol by stomach tube. At the end of 4 hours the animals were sacrificed and the intestinal tracts removed. This time interval was chosen, since in the previous experiments it was found that at the end of this period the vitamin A level in the intestinal wall had attained its maximum value. Although vitamin A

\* Aided by a grant from the Nutrition Foundation, Inc. The author wishes to express his appreciation for the use of the facilities of the Hancock Foundation. Contribution No. 185 from the Department of Biochemistry, University of Southern California.

<sup>1</sup> Mattson, F. H., Mehl, J. W., and Deuel, H. J., Jr., *Arch. Biochem.*, 15, 65 (1947).

<sup>2</sup> Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

is also present in the liver at that time, the amount in the intestinal wall exceeds that in the liver. The contents of the intestines were flushed out with 0.9 per cent sodium chloride and the intestines combined into one sample. The sample was saponified with alcoholic KOH and extracted with Skellysolve A. The petroleum ether was then extracted three times with 90 per cent methyl alcohol. In this way most of the vitamin A is extracted by the methyl alcohol, leaving the carotene in the Skellysolve. Sufficient water was then added to the methyl alcohol solution to reduce the concentration of alcohol to approximately 50 per cent. This was then extracted with Skellysolve B. The Skellysolve was dried with anhydrous sodium sulfate and reduced to a small volume in a stream of nitrogen. This was placed on a column of 1:1 magnesium oxide-Hyflo and the chromatogram developed with Skellysolve B. The fluorescing band was separated mechanically and eluted with Skellysolve B containing a small amount of ethyl alcohol. The absorption curve of this fluorescing band was determined on the Beckman spectrophotometer. These results with those determined on vitamin A alcohol<sup>3</sup> are shown in the graph. A mixed chromatogram of vitamin A alcohol and the fluorescent material isolated from the intestines gave a single fluorescing band on a column of magnesium oxide and Hyflo with Skellysolve B as the solvent and developing agent.

Since the material isolated from the intestines of the rats exhibited fluorescence, possessed an absorption curve similar to that of vitamin A, and showed a single fluorescing band in a mixed chromatogram with vitamin A, it is reasonably certain that vitamin A is present in the intestinal wall of rats 4 hours after supplementing with carotene.

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Received for publication, October 4, 1948

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<sup>3</sup> Obtained from Distillation Products, Inc., Rochester, New York.

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# OXIDATION IN VIVO OF EMULSIFIED RADIOACTIVE TRILAURIN ADMINISTERED INTRAVENOUSLY\*

Sirs:

Studies conducted in this laboratory have clearly shown that fat injected intravenously in an emulsified form is utilized for energy by the dog.<sup>1</sup> The rate of disappearance of such fat from the blood of various species has been found to be very rapid.<sup>2</sup> In order to determine how rapidly such fat is metabolized *in vivo*, use has been made of an emulsion which contained radioactive trilaurin. The radioactive lauric acid present

*Excretion of C<sup>14</sup>O<sub>2</sub> by Rat following Intravenous Injection of  
Emulsified Trilaurin (—C<sup>14</sup>OO—)*

Sample No.	Time of collection		Specific activity*	Injected C <sup>14</sup> activity expired
	Per sample	Total		
	<i>min.</i>	<i>min.</i>		<i>per cent</i>
1	10	10	4100	2.76
2	10	20	7560	7.23
3	15	35	9060	15.77
4	20	55	7910	25.39
5	35	90	7220	40.01
6	40	130	5510	51.31
7	35	165	3580	58.43
8	35	200	2640	63.78
9	35	235	2050	67.18
10	35	270	1670	70.85

\* Specific activity = counts per minute per mg. of CO<sub>2</sub> carbon.

in the trilaurin contained C<sup>14</sup> in the carboxyl group, and the trilaurin was made by direct esterification.<sup>3</sup> An emulsion was made containing 0.6 gm. of coconut oil, 0.4 gm. of the trilaurin, water, dextrose, and stabilizers, and made up to a total volume of 20 ml. This product was sealed in glass ampules under N<sub>2</sub> and sterilized by autoclaving.

Although a number of metabolic studies have been carried out, only one typical example will be described here. A non-fasted female rat

\* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., the Milbank Memorial Fund, The Upjohn Company, and the National Dairy Council.

<sup>1</sup> McKibbin, J. M., Ferry, R. M., Jr., and Stare, F. J., *J. Clin. Invest.*, **25**, 679 (1946).

<sup>2</sup> Geyer, R. P., Mann, G. V., and Stare, F. J., *J. Lab. and Clin. Med.*, **33**, 175 (1948).

<sup>3</sup> Details concerning the syntheses and emulsification techniques will be described in a more complete paper now in preparation.

weighing 270 gm. was anesthetized lightly with ether and was injected intravenously through a tail vein with 3.9 ml. of the above emulsion. The animal was immediately placed in a respirometer chamber and serial samples of expired  $\text{CO}_2$  were collected in 1 N NaOH. Total  $\text{CO}_2$  determinations were made by the Van Slyke manometric technique, and radioactivity assays were made on  $\text{BaCO}_3$  samples prepared from the alkali-carbonate solution. Preparation of the samples and counting were accomplished according to the method described by Olson *et al.*<sup>4</sup> The results are given in the accompanying table.

Essentially the same results were obtained with animals fasted 24 hours. From these data it is apparent that properly emulsified fat is utilized immediately for energy following its intravenous administration.

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Received for publication, October 14, 1948

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<sup>4</sup> Olson, R. E., Miller, O. N., Topper, Y. J., and Stare, F. J., *J. Biol. Chem.*, **175**, 503 (1948).

## 2,6-DIAMINOPURINE, A PRECURSOR OF NUCLEIC ACID GUANINE\*

Sirs:

2,6-Diaminopurine (2-aminoadenine), which was postulated<sup>1</sup> as one of the possible intermediates involved in the conversion of adenine into nucleic acid guanine,<sup>2</sup> has been synthesized<sup>3</sup> with isotopic nitrogen in the 1

	Atom per cent excess N <sup>15</sup> *		Calculated on basis of 100 per cent N <sup>15</sup> in purine fed
2,6-Diaminopurine (dietary)	5.44	5.45	100
Sodium nucleic acids	0.076	0.075	
Copper purines	0.148	(0.157†)	
Adenine (calculated from picrate)‡		0.043	
“ (free)‡		0.042	0.78
Guanine sulfate‡		0.222	
“ (free)‡		0.216	4.0
Silver pyrimidines	0.022		0.40
Allantoin	0.336	0.335	6.2
Urea	0.023	0.023	0.42
Ammonia	0.020	0.022	0.38
Total urinary nitrogen	0.051	0.054	0.96

\* Consolidated-Nier ratio mass spectrometer; duplicates are on independent digestions and conversions.

† Insufficient gas sample for optimum determination.

‡ The guanine was shown to be free of adenine by paper chromatography (Vischer, E, and Chargaff, E., *J. Biol. Chem.*, **176**, 703 (1948)). The adenine contained a trace of foreign material, not definitely identified as guanine

and 3 positions of the ring and in the 2 amino group. It was administered by stomach tube to rats at a level of 29 mg. per kilo per day for 3 days.

\* The authors wish to acknowledge the assistance of the James Foundation of New York, Inc., the National Cancer Institute of the United States Public Health Service, and the Office of Naval Research.

<sup>1</sup> Brown, G. B., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **13**, in press

<sup>2</sup> Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172**, 469 (1948).

<sup>3</sup> Bendich, A., Tinker, J. F., and Brown, G. B., *J. Am. Chem. Soc.*, **70**, 3109 (1948).

The urinary constituents and the total nucleic acids of the viscera were examined (see the table).

The results indicate that 2,6-diaminopurine is an effective precursor of nucleic acid guanine.

It is interesting that both adenine and 2,6-diaminopurine are toxic for rats at levels at which other purines have proved to be non-toxic, and that 2,6-diaminopurine evinces *apparent* "antipurine-metabolite" behavior<sup>4</sup> under certain conditions. In so far as the authors are aware, the presence of this purine has not been detected in nature.

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Received for publication, October 15, 1948

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<sup>4</sup>Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A., *J. Biol. Chem.*, **174**, 765 (1948).

# GLUTAMINE AND THE GROWTH OF STAPHYLOCOCCUS AUREUS

Sirs:

Grossowicz<sup>1</sup> recently reported experiments from which he concluded that the addition of 0.6 to 2.0 mg. of L-glutamine per ml. of medium (supplemented casein hydrolysate) inhibited the growth of *Staphylococcus aureus*, and that this inhibition was abolished by L-glutamate. Inhibitions by commercial preparations of L-glutamine had been observed in this

Growth of *Staphylococcus aureus* on the medium of Lichtenstein and Grossowicz\* at 37°. Inoculum 10<sup>6</sup> organisms from 16 hour broth culture. The L-glutamate and L-glutamine were sterilized by filtration of a 1 per cent stock solution. 0 indicates no growth, + visible growth, ++++ maximum growth.

Substances added to 5 ml medium	Growth		
	16 hrs	24 hrs.	48 hrs.
None.	+	++	++++
L-Glutamine (British Drug Houses), 10 mg.	0	0?	++++
“ “ “ “ purified), 2-40 mg.	+	+++	++++
L-Glutamate, 2 mg.	0?	++	++++
Zn (as ZnCl <sub>2</sub> ), 0.001 mg.	0	0	0
“ “ “ 0.005 “	0	0	0?
“ “ “ 0.001 “ L-glutamate, 2 mg.	0	++	++++
“ “ “ 0.005 “ “ 2 “	0	+++	++++
“ “ “ 0.01 “ L-glutamine, 10 mg.	0	++	++++
“ “ “ 0.005 “ “ 10 “	0?	+++	++++

\* Lichtenstein, N., and Grossowicz, N., *J. Biol. Chem.*, 171, 387 (1947).

laboratory some time ago during work on streptococci, but no inhibition occurred when pure glutamine was used. Two strains of *Staphylococcus aureus* showed results similar to those of Grossowicz when a preparation of L-glutamine from The British Drug Houses (Batch 639261) was used. This specimen was purified by passing H<sub>2</sub>S through a slightly alkaline solution and then treating it according to Archibald.<sup>2</sup> Concentrations up to 8.0 mg. per ml. of the purified material did not inhibit growth. The British Drug Houses examined the above batch of impure glutamine polarographically, and found 0.75 per cent Zn and 0.44 per cent Ba. The amide nitrogen was 54 per cent of the theoretical value. Other impurities

<sup>1</sup> Grossowicz, N., *J. Biol. Chem.*, 173, 729 (1948).

<sup>2</sup> Archibald, R. M., *J Biol Chem.*, 159, 693 (1945).

were arginine (2 per cent), ammonium pyrrolidone carboxylate, and ammonium glutamate. Zn in concentrations comparable to those present in impure glutamine inhibited growth completely; this inhibition was reversed by L-glutamate and L-glutamine (see the table).

As Grossowicz used commercial preparations of glutamine, one of which was supplied by The British Drug Houses, it is probable that the inhibitions observed by him were due to impurities.

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Received for publication, October 26, 1948

# THE PARTICIPATION OF INORGANIC PYROPHOSPHATE IN THE REVERSIBLE ENZYMATIC SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE

Sirs:

A purified enzyme preparation has been obtained from an autolysate of dried brewers' yeast by ammonium sulfate fractionation and isoelectric precipitation which catalyzes the reaction: nicotinamide mononucleotide (NMN) + adenosine triphosphate (ATP)  $\rightleftharpoons$  DPN + inorganic pyrophospho-

Substance estimated†	DPN synthesis,* micromoles per ml.			ATP synthesis,* micromoles per ml.		
	0 min.	60 min.	$\Delta$	0 min.	60 min.	$\Delta$
NMN‡	2.50			0.0		
ATP	2.20	1.36	-0.84	0.0	1.02	+1.02
DPN	0.0	0.74	+0.74	1.50	0.61	-0.89
P-P	0.0	0.83	+0.83	1.80	0.79	-1.01
Orthophosphate	0.11	0.13		0.16	0.09	
Phosphate, acid-labile	4.44	4.50		3.60	3.66	

\* For DPN synthesis, 1.0 ml. of reaction mixture contained 50  $\gamma$  of the enzyme preparation, 0.3 micromole of  $MgCl_2$ , and 50 micromoles of glycylglycine buffer (pH 7.4) in addition to ATP and NMN; for ATP synthesis 1.0 ml. contained 50  $\gamma$  of the enzyme preparation, 0.75 micromole of  $MgCl_2$ , and 50 micromoles of glycylglycine buffer (pH 7.4) in addition to DPN and P-P. Constant values were reached after 30 to 40 minutes at 38°.

† ATP was estimated spectrophotometrically by triphosphopyridine nucleotide reduction in the presence of glucose, hexokinase, and *Zwischenferment*, and DPN by reduction with the triose phosphate dehydrogenase system. Inorganic pyrophosphate was estimated as orthophosphate after acid hydrolysis of the precipitated and washed manganous salt and acid-labile phosphate as the orthophosphate released after 10 minute hydrolysis in 1  $NH_2SO_4$  at 100°.

‡ NMN was prepared by hydrolysis of DPN with nucleotide pyrophosphatase.<sup>1 2</sup> After purification the ratio, nicotinamide-ribose moiety to organic phosphate, was 1.0.

phate (P-P). In the table are summarized two experiments in which equilibrium was attained starting from the left (DPN synthesis) and the right (ATP synthesis). The equilibrium constant,  $K = ((DPN)(P-P))/((NMN)(ATP))$ , calculated from the data of the two experiments is 0.3 in one case and 0.5 in the other. The concentrations of acid-labile phosphate were unchanged and no orthophosphate was produced. Nicotinamide nu-

<sup>1</sup> Kornberg, A., *J. Biol. Chem.*, **174**, 1051 (1948).

<sup>2</sup> Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **176**, 665 (1948).

cleoside, adenosine diphosphate, and adenylic acid were inactive in DPN synthesis. The reduced form of DPN was split by the purified enzyme preparation in the presence of inorganic pyrophosphate, but triphosphopyridine nucleotide and flavin-adenine dinucleotide were not. The possibility that the latter two nucleotides may participate in analogous reactions with crude enzyme preparations requires further study.

These findings indicate a mechanism for the synthesis of DPN and for the origin and function of inorganic pyrophosphate. Ochoa, Cori, and Cori<sup>3</sup> isolated inorganic pyrophosphate from dialyzed rat liver dispersions in which glutamate, pyruvate, or succinate was being oxidized. It was later identified in washed rabbit kidney particles oxidizing glutamate,<sup>4,2</sup> in molds,<sup>5</sup> and in yeast.<sup>6</sup> The present findings suggest that the accumulation of inorganic pyrophosphate in fungi and in tissues may be explained by a sequence of three reactions: (1) the irreversible hydrolysis of DPN by nucleotide pyrophosphatase<sup>1, 2</sup> to yield NMN and adenylic acid, (2) the phosphorylation of adenylic acid to ATP in respiration or fermentation, and (3) the combination of NMN with ATP to produce inorganic pyrophosphate and regenerate DPN.

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Received for publication, November 10, 1948

<sup>3</sup> Cori, C. F., in A symposium on respiratory enzymes, Madison (1942).

<sup>4</sup> Green, D. E., *et al.*, Abstracts, American Chemical Society, Atlantic City, 26E April (1947).

<sup>5</sup> Mann, T., *Biochem. J.*, **38**, 345 (1944).

<sup>6</sup> Lindahl, P. E., and Lindberg, O., *Nature*, **157**, 335 (1946).

<sup>7</sup> The valuable technical assistance of Mr. W. E. Pricer, Jr., is gratefully acknowledged.



# RELATION OF VITAMIN B<sub>12</sub> TO THE GROWTH FACTOR PRESENT IN COW MANURE

Sirs:

Crystalline vitamin B<sub>12</sub>, reported by Ott *et al.*<sup>1</sup> to have activity for chick growth, has been found to be completely effective, either orally or by intramuscular injection, when tested by the method developed in this laboratory<sup>2</sup> to assay quantities of the unknown growth factor occurring in cow manure, in fish-meal, and in some other feedstuffs of animal origin. Details

Experiment No.	Supplement	Level of supplement in diet, $\gamma$ per 100 gm.	No. of chicks	Average gain, 2 to 4 wks. of age gm.
1	None		9	40.6
	Acid ppt.	75,000	9	77.3
	" "	150,000	8	89.1
	" "	300,000	8	94.9
	Vitamin B <sub>12</sub> (crystalline)*	0.25	9	62.6
	" " "	0.50	9	81.7
	" " "	1.00	8	85.4
	" " "	2.00	9	100.9
		Single injection of supplement, $\gamma$ per chick		
2	None		8	89.3
	2 unit liver extract†	100,000	9	98.3
	2 " " "	200,000	8	115.0
	2 " " "	300,000	9	116.6
	Vitamin B <sub>12</sub> (crystalline)	1.25	9	123.4
	" " "	2.5	9	123.3
	" " "	5.0	9	120.4

\* Prepared by Merck and Company, Inc., Rahway, New Jersey.

† Liver injection (crude), 2 U. S. P. units per ml.

of the method were the same as those reported previously,<sup>2</sup> except that the progeny of undepleted hens were used in the second of the two experiments. Injections were made into the breast muscle on the 1st day of the 14 day experimental period.

The results in the table show that the maximum growth response was the same in Experiment 1 for crystalline vitamin B<sub>12</sub> and the acid precipi-

<sup>1</sup> Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 174, 1047 (1948).

<sup>2</sup> Bird, H. R., Rubin, M., and Groschke, A. C., *J. Biol. Chem.*, 174, 611 (1948).

tate of water extract of cow manure, and essentially the same in Experiment 2 for the crystalline vitamin and 2 unit liver extract. The gains obtained in Experiment 1 were plotted against the dietary level of the crystalline vitamin and a dose-response curve constructed. The gain obtained with the lowest level of acid precipitate was applied to the straight line portion of the curve, and it was calculated that the acid precipitate contained the equivalent of 5.8  $\gamma$  of vitamin B<sub>12</sub>. Comparative chick and bacterial assays would be of interest as a means of determining the possible existence of different forms of the vitamin.

In view of the potency of this vitamin as a bacterial growth factor the injection experiments are of particular interest since they show that its effect on the chick is direct and not mediated through the intestinal flora.

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Received for publication, October 23, 1948

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